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A MODIFIED SPECTROPHOTOMETRIC DETERMINATION OF CHYMOTRYPSIN, TRYPSIN, AND THROMBIN[1]

BRIAN C. W. HUMMEL

Abstract

The spectrophotometric procedure proposed by Schwert and Takenaka for the assay of chymotrypsin and trypsin has been modified and extended to include the application to N-benzoyl-L-tyrosine ethyl ester and α-*p*-toluenesulphonyl-L-arginine methyl ester. The greater degree of sensitivity and specificity thus achieved permits the determination of traces of chymotrypsin in the presence of relatively large amounts of trypsin and vice versa. A similar spectrophotometric procedure for the assay of thrombin is described.

Introduction

The well-known Schwert and Takenaka method (1) for the assay of trypsin and chymotrypsin is based upon the change that occurs in ultraviolet absorbance during the hydrolysis of amino acid esters by these enzymes. The change in difference between the absorbance of the ester and that of the corresponding acid during hydrolysis of the ester is measured at the wavelength of maximum difference. The substrate α-benzoyl-L-arginine ethyl ester (BAEE) together with α-benzoyl-L-arginine (BA) as blank were used for trypsin, while the substrates L-tyrosine ethyl ester or N-acetyl-L-tyrosine ethyl ester (ATEE) together with tyrosine or N-acetyl-L-tyrosine, respectively, were used for chymotrypsin.

Although this method is one of the most satisfactory[2] so far proposed for the assay of proteolytic enzymes, it has certain practical limitations: (*a*) two solutions (acid and ester) must be prepared; (*b*) in the case where the acid, i.e. BA absorbs ultraviolet light more strongly than the corresponding ester (BAEE), the spectrophotometer must be balanced by opening the entrance slit during the period of hydrolysis, thus limiting undesirably the maximum substrate concentration which may be used without a photomultiplier; (*c*) BAEE is slowly hydrolyzed by chymotrypsin (3) rendering this substrate unsuitable for the assay of traces of trypsin in the presence of a relatively large amount of chymotrypsin; (*d*) with the substrates employed previously (1), advantage is not taken of the greater sensitivity of the method which would be available

[1]Manuscript received June 17, 1959.
Contribution from Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.

[2]While the spectrophotometric method of Martin *et al.* (2) using the substrate N-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester is extremely sensitive, it is not specific for chymotrypsin.

if the substrate *p*-toluenesulphonyl-L-arginine methyl ester (TAME) were used in place of BAEE, and benzoyl-L-tyrosine ethyl ester (BTEE) in place of ATEE. In the interest of greater convenience, sensitivity, and specificity, certain modifications of the Schwert and Takenaka method have been made and the latter has been applied also to the estimation of thrombin, whose specificity is very similar to that of trypsin (4).

In the procedure presented here TAME is employed as a substrate for trypsin and thrombin while BTEE is used with chymotrypsin. TAME offers two advantages over BAEE in that it is more rapidly hydrolyzable by trypsin (3) and it is not hydrolyzed by chymotrypsin (3). It is thus suitable for the assay of traces of trypsin in the presence of large amounts of chymotrypsin. BTEE is more satisfactory than ATEE as a substrate for the assay of chymotrypsin in the presence of relatively large amounts of trypsin since, under the conditions described below, BTEE is more susceptible to chymotryptic hydrolysis and has apparently complete resistance to tryptic hydrolysis.

In addition to using substrates other than those employed previously (1), the method has been modified by placing the substrate in both cuvettes, thus eliminating the necessity of using the acylated amino acid as blank.[3] With this arrangement, two possibilities exist: (*a*) in the ultraviolet region the hydrolysis products absorb more strongly than does the substrate, or (*b*) the reverse is true. In case (*a*) the spectrophotometer is set to zero with the control cuvette in the light path, while in case (*b*) it is set to zero with the reaction cuvette in the light path. In either case, the absorbance difference is initially zero and increases during the course of hydrolysis. In bringing the instrument to zero before each reading the slit is kept nearly constant in width during the experiment (case (*a*)), or is slightly narrowed each time (case (*b*)). Thus it is never necessary to *increase* the slit width from its initial setting, as was previously the case (1). In the procedure given below, the substrate concentration used was the highest permissible with the sensitivity and slit width settings of the spectrophotometer near the maximum. With substrate concentrations thus limited to 0.5 to 1.0×10^{-3} *M* it was found possible to obtain zero order kinetics over a range of absorbance readings suitable for assay purposes without the photomultiplier. Where it is desired to employ higher substrate concentrations, as in the determination of kinetic constants, a photomultiplier is required.

Materials and Methods

Amino Acid Derivatives[4]

(*a*) *α-p*-Toluenesulphonyl-L-arginine methyl ester hydrochloride (TAME. HCl) (m.p.:[5] found 146–149°, reported 146°(5)) was obtained from Mann Research Laboratories Inc. (lot No. 105641) and used without further purification. It migrated as a single component when chromatographed on paper using butanol – acetic acid – water (4:1:5 v/v, upper phase) (6) and sprayed with a Sakaguchi (7) reagent.

[3]This modification was suggested by Dr. J. A. Drum.
[4]These compounds did not give a blue color with ninhydrin.
[5]All melting points are corrected.

(*b*) α-*p*-Toluenesulphonyl-L-arginine hydrochloride (TA.HCl), was prepared by the method used by Clarke and Gillespie (8) for the preparation of the corresponding benzenesulphonyl derivative. To 5.34 g (25 mmoles) of L-arginine monohydrochloride and 11.0 g (80 mmoles) of potassium carbonate dissolved in 30 ml of water were added 7.42 g (39 mmoles) of *p*-toluenesulphonyl chloride dissolved in ether. The mixture was stirred vigorously at room temperature for 2½ hours and allowed to stand overnight. Some solid matter separated. Ether and water were added and the mixture again stirred vigorously to promote crystallization. The crystals were recovered by filtration, washed with water and ether, and allowed to dry on the filter. An 8.2-g yield (94% of theoretical) was obtained. Recrystallization was carried out using water as solvent. Separation of the desired product from unreacted starting materials, *p*-toluenesulphonate and the N,N′-di-*p*-toluenesulphonyl derivative was achieved by passing an aqueous solution of the crystals through a column of IR-120 cation exchange resin (obtained from Rohm and Haas Co.) in the hydrogen form. Arginine and its *p*-toluenesulphonyl derivatives were retained on the column while anions were washed through. The column was eluted with 1 *N* hydrochloric acid and fractions which gave a positive Sakaguchi reaction but a negative ninhydrin reaction were collected. The desired product was recovered by evaporation of the solvent and crystallization as before, m.p.: found 257–259° (for the hydrochloride), reported 256–257°d (9) (for the acetate).

(*c*) N-benzoyl-L-tyrosine ethyl ester (BTEE) (m.p.: found 112–118°, reported 120–121° (10)), obtained from Mann Research Laboratories (lot No. 105635), was used without further purification. A single component was found by paper chromatography with the solvent system described above and with diazotized sulphanilic acid as the spray reagent.

(*d*) N-benzoyl-DL-tyrosine (BT) was prepared from O,N-dibenzoyl-DL-tyrosine obtained from Mann Research Laboratories (lot No. 2624). The O-benzoyl group was removed with sodium hydroxide at room temperature (11) and the melting point of the product was found to be 186–192° (reported 191–193° (12)).

(*e*) N-*p*-toluenesulphonyl-L-tyrosine ethyl ester (TTEE) was prepared from recrystallized tyrosine ethyl ester hydrochloride (Eastman Kodak No. 6671) by the method of Fischer and Lifschitz (13); the melting point of the product was found to be 113–114.5°; reported 114° (11). Found $[\alpha]_D^{25}$ +11.1 in alcohol; reported $[\alpha]_D^{17}$ +6.76 (13) in alcohol.

(*f*) N-*p*-toluenesulphonyl-L-tyrosine (TT) was prepared from the ethyl ester prepared in (*e*) by the method of Fischer and Lifschitz (13); m.p.: found 181–184°, reported 187–188° (13).

Buffer Solutions

(*a*) For chymotrypsin assay: A solution of 10.55 g of $CaCl_2.2H_2O$ in 250 ml of 0.2 *M* tris(hydroxymethyl)aminomethane ("Sigma 7-9 Buffer", Sigma Chemical Co.) was adjusted to pH 7.8 with hydrochloric acid and diluted to 1 liter. To this solution was added 432 ml of methanol, making the final buffer 25.6% methanol (w/w) and 0.05 *M* in calcium (14).

(*b*) For trypsin and thrombin assay: A solution of 1.47 g of $CaCl_2.2H_2O$ dissolved in 200 ml of 0.2 *M* tris(hydroxymethyl)aminomethane ("Sigma 7-9 Buffer") was adjusted to pH 8.1 with hydrochloric acid and diluted to 1 liter. The calcium concentration was then 0.01 *M* (15, 16).

Substrate and Blank[6] *Solutions*

(*a*) For chymotrypsin (using buffer (*a*) above): first substrate (5×10^{-4} *M*): 15.7 mg BTEE/100 ml solution; first blank (5×10^{-4} *M*): 14.3 mg BT/100 ml solution; second substrate (10^{-3} *M*): 18.2 mg TTEE/50 ml solution; second blank (10^{-3} *M*) 16.8 mg TT/50 ml solution.

(*b*) For trypsin and thrombin (using buffer (*b*) above): substrate (1.04×10^{-3} *M*): 19.7 mg TAME.HCl/50 ml solution; blank (1.04×10^{-3} *M*): 19.0 mg TA.HCl/50 ml solution.

Enzyme Solutions

Both chymotrypsin[7] (crystalline salt-free lot No. 105498) and trypsin[7] (twice-crystallized salt-free lot No. 105496), were obtained from Mann Research Laboratories Inc. Thrombin (lot No. 032005B) was obtained from Parke Davis and Company.

Assay Procedure

A Beckman DU spectrophotometer, fitted with thermospacers maintained at $30\pm0.1°$ C, was used. Measurements were made using quartz cells with 1-cm light paths and volumes of approximately 4 ml. Exactly 3.00 ml of substrate were placed in each cuvette. To the first cuvette (control) was added approximately 150 μl of water accurately measured with a micropipette, and to the second was added an equal volume of a solution of enzyme in water. After each addition the contents of each cuvette were agitated for 5–10 seconds with a small spatula. Absorbance differences (initially zero) were measured at 30-second intervals.

Results and Discussion

Differential Spectra

The absorbance spectrum of 3.8×10^{-4} *M* BT with respect to BTEE at the same concentration (blank) is shown in Fig. 1A. Both substances were dissolved in the pH 7.8 buffer used for chymotrypsin assay. A maximum occurs at 254.0 mμ.

The difference spectrum of 6.7×10^{-4} *M* TT with reference to TTEE at the same concentration is shown in Fig. 1B. A maximum occurs at 248.0 mμ. The solvent was the same as for BT and BTEE.

A similar differential spectrum shown in Fig. 1C was obtained when the absorbance of 3.8×10^{-4} *M* TA solution was measured with respect to TAME at the same concentration. In this case a maximum occurs at 244.0 mμ, the solvent being the pH 8.1 buffer used for trypsin assay.

[6]Used in the determination of difference spectra only (Fig. 1).

[7]Extinction coefficients measured in 10^{-3} *M* HCl: 1.57/cm/mg/ml at 280 mμ for trypsin and 1.85/cm/mg/ml at 282 mμ (cf. (1)).

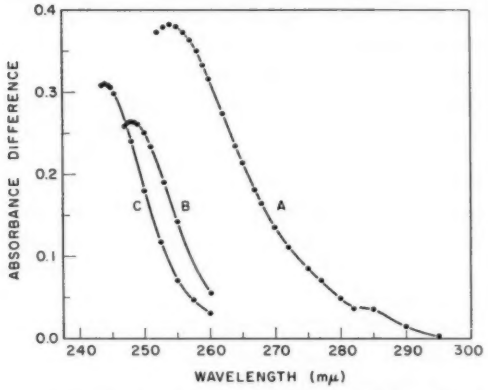

FIG. 1. Differential spectra: A: BT vs. BTEE, both 3.75×10^{-4} *M* at pH 7.8; B: TT vs. TTEE, both 6.67×10^{-4} *M* at pH 7.8; C: TA vs. TAME, both 3.90×10^{-4} *M* at pH 8.1.

Enzyme Activity Measurements

(*a*) Chymotrypsin: Measurements were made at 256 mμ. A linear rate of increase of absorbance occurred up to a reading of 0.13, corresponding to approximately 31% hydrolysis. The initial rate of hydrolysis was proportionate to enzyme concentration in the range of 0.5–3.0 μg/ml (Fig. 2).

The specific rate of hydrolysis of BTEE by chymotrypsin was found to be 3.8×10^{-3} absorbance units/min/μg enzyme/ml or 0.14 μmoles/min/μg enzyme/ml. Under the same conditions, acetyl-L-tyrosine ethyl ester (ATEE, 10^{-3} *M* Mann lot No. 1744) was hydrolyzed at approximately one eighth the

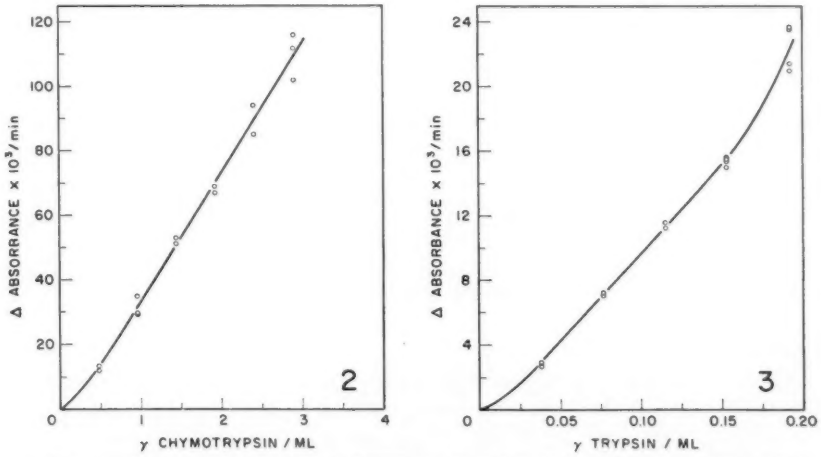

FIG. 2. The rate of hydrolysis of BTEE as a function of chymotrypsin concentration at 30° in pH 7.8 tris–HCl buffer containing methanol (25.6% w/w) and calcium chloride (0.05 *M*).

FIG. 3. The rate of hydrolysis of TAME as a function of trypsin concentration at 30° in pH 8.1 tris–HCl buffer containing calcium chloride (0.01 *M*).

rate observed for BTEE. With trypsin (50 μg/ml pH 7.8 buffer) no hydrolysis of BTEE was observed, whereas ATEE was hydrolyzed at the rate of 1.8×10^{-3} absorbance units (measured at 238 mμ)/min.

(*b*) Trypsin: Measurements were made at 247 mμ. A linear rate of increase of absorbance occurred up to a reading of 0.24, corresponding to approximately 65% hydrolysis. The initial rate of hydrolysis by trypsin was found to be directly proportionate to enzyme concentration in the range 0–0.15 μg/ml (Fig. 3). The specific rate of hydrolysis of TAME by trypsin was found to be 101×10^{-3} absorbance units/min/μg enzyme/ml or 0.86 μmoles/min/μg enzyme/ml with 0.15 μg enzyme/ml. The results of 11 assays carried out in duplicate on the same sample showed a standard deviation of 2.1%. No hydrolysis of TAME by chymotrypsin could be detected, a finding in accord with that of Schwert *et al.* (3).

(*c*) Thrombin: Absorbance measurements were made at 247 mμ. A linear rate of increase of absorbance occurred up to a reading of 0.19, corresponding to approximately 51% hydrolysis. The initial rate of hydrolysis was proportionate to the enzyme concentration up to approximately 20 μg/ml but, with higher concentrations, the slope of the curve increased (Fig. 4). The specific rate of hydrolysis of TAME was found to be 0.44×10^{-3} absorbance units/min/μg enzyme/ml or 0.0038 μmoles/min/μg enzyme/ml with 20 μg enzyme/ml.

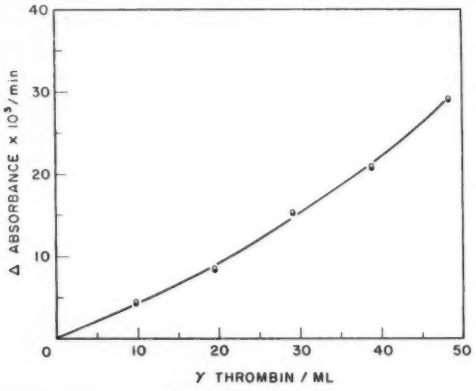

FIG. 4. The rate of hydrolysis of TAME as a function of thrombin concentration at 30° in pH 8.1 tris–HCl buffer containing calcium chloride (0.01 *M*).

Hydrolysis of TTEE

The hydrolysis of N-*p*-toluenesulphonyl-L-tyrosine ethyl ester was studied to determine whether this substrate would prove to be more susceptible to attack by chymotrypsin than is BTEE. This, however, was not the case; it was found, furthermore, that TTEE is not a suitable substrate for use in chymotrypsin assay since the time course of its hydrolysis, even at a concentration of 4×10^{-3} *M*, indicates that the zero order reaction did not persist long enough to make precise assay feasible. The kinetics of hydrolysis of TTEE, which do

not show the characteristics of simple product inhibition(17), will be the subject of a later publication.

Acknowledgment

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ANTIGENIC PROPERTY AND ELECTROPHORETIC BEHAVIOR OF THE SERUM ALBUMIN OF RATS BEARING THE WALKER TUMOR[1]

D. Dufour, L. Berlinguet, and J. M. Loiselle

Abstract

The serum of Walker tumor-bearing rats contains antigens which give an immunological response with rabbit anti-Walker antibodies. These antigens are located in the albumin fraction of the serum. The albumins of the normal and cancerous rats have different migration rates during electrophoresis and different levels of polysaccharides.

Introduction

It was shown in an earlier publication (1) that the total serum of Walker tumor-bearing rats has an antigenic property. This property was evidenced by a positive anaphylactic reaction in guinea pigs, according to a method recommended by Ovary and Bier (5).

It has also been shown by Berlinguet, Dufour, and Gagné (2), by Balis *et al.* (3), and by Bozetti and Magrassi (4) that, in tumor-bearing rats, the ratio of serum proteins is markedly changed.

It was then interesting to find out a possible relationship between this antigenic property and the fluctuations of the serum proteins.

By the use of the Ovary and Bier test, an assay of localization of the antigenic property has been attempted and each electrophoretic protein fraction of the serum of Walker tumor-bearing rats has been analyzed for an antigenic property in guinea pigs.

Experimental

Immune Serum Preparation

Rats bearing a Walker tumor were killed 14 days after the implantation of the tumor. The tumor was dissected, suspended in 10 times its weight of 0.9% saline solution, and homogenized. It was then lyophilized *in vacuo*. The weight of the residue was taken and enough sterile physiological saline solution was added to obtain a concentration of 15 mg of dry residue per milliliter of suspension. One milliliter of this lyophilized Walker tumor preparation was given daily during a 30-day period to a rabbit. The site of injection was alternatively intramuscular for 1 week and subcutaneous for the next week. At the end of that period, the rabbit was sacrificed and the total serum was subjected to lyophilization. The dried residue was dissolved in sterile physiological saline solution. The final concentration was 50 mg of powdered serum per milliliter of solution.

Preparation of Serum Protein Fractions

A 14-day-old Walker tumor was dissected and weighed. It was then homogenized. Enough 0.9% saline solution was then added to the residue to obtain

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Contribution from the Department of Biochemistry, Laval University, Quebec, Que. This work has been made possible owing to a grant generously given by the National Cancer Institute of Canada.

a final concentration of 1 g of the wet weight of the tumor per 10 ml of solution. One milliliter of this tumor preparation was given by intramuscular injections to five young rats weighing about 100 g. The growth of this tumor is very rapid and after 2 weeks, the average weight is about 19 g and the tumor always takes in each animal. Seven days after the transplantation, the rats were killed, the serum was pooled and lyophilized; the serum of five normal control rats was treated the same way. The 7-day period was chosen because at that time the tumor is well established and catabolic necrosis has not yet taken place.

After lyophilization, 50 mg of serum powder was dissolved in 10 ml of isotonic saline and a volume of 0.02 ml was used for paper electrophoresis. Once dried, the paper strip was divided longitudinally into a wide portion and a narrow one. The narrow portion was dyed with bromophenol blue and used as a guide in the localization of the protein fractions on the wide, untouched portion of the paper strip. Each fraction was then isolated by means of scissors and the sectioned papers were cut in small pieces and eluted in 10 ml of isotonic saline. The resulting solution was filtered and the clear filtrate assayed for antigenic property.

Local Anaphylactic Test for Antigenicity

Guinea pigs were used according to the original procedure recommended by Ovary and Bier (5). A volume of 0.1 ml of the immune serum, prepared as described above, was injected into the skin of guinea pigs and allowed to diffuse during an 18-hour period. (A period of 18 hours is considered sufficient to obtain a high degree of diffusion.)

Then 0.50 ml of the serum protein fraction obtained by the method described above was mixed with 0.25 ml of Evans blue 5:1000 solution in saline and was given intravenously. After 15 minutes, the guinea pigs were killed and the skin was examined at the site of injection. An intense blue color was considered as a positive reading. The absence of color was a negative reading.

Electrophoretic Conditions

The paper electrophoresis was done using a current of 2 ma during a period of 20 hours in a barbital buffer 0.05 *M*, at a pH of 8.6.

Determination of the Polysaccaride Level of the Albumins

The albumin was precipitated from the serum by addition of a 0.64 saturated solution of ammonium sulphate. After a few hours, the precipitate was collected by filtration and dried at room temperature during 24 hours.

The polysaccharides were estimated according to the method of Fluger modified by Somogyi (9). After hydrolysis of the polysaccharides, the glucose was analyzed according to the method recommended by Nelson (10) and the results expressed as milligrams of glucose per 100 g of dry albumin.

Results

Figure 1 demonstrates how the Ovary and Bier test has revealed the presence of a reaction between the serum of Walker tumor-bearing rat and the anti-Walker tumor antibodies produced in a rabbit. The skin of the guinea pig

PLATE I

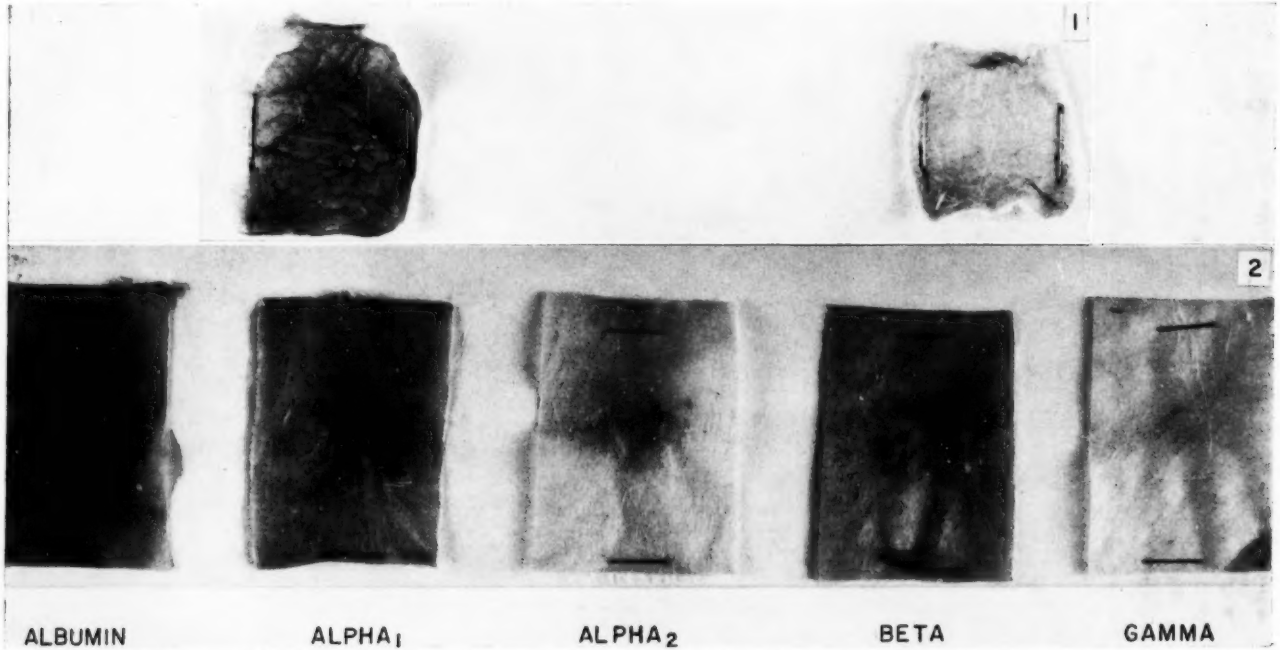

FIG. 1. Positive anaphylactic reaction (blue color on the skin at left) in guinea pigs sensitized with 5 mg of powdered serum containing antibodies to Walker tumor and treated with 10 mg of serum powder obtained from a Walker tumor-bearing rat.

No color (right) on the skin of guinea pigs also sensitized with 5 mg of powdered serum containing antibodies to Walker tumor and treated with 10 mg of serum powder obtained from a normal rat.

FIG. 2. Positive anaphylactic reaction (blue color of the skin at far left) in guinea pigs sensitized with 5 mg of powdered serum containing antibodies and treated with the albumin fraction, electrophoretically separated from the lyophilized serum of Walker tumor-bearing rats. The globulin fraction as a whole lead to negative anaphylactic reaction. (Absence of blue color on the four skins at right.)

PLATE II

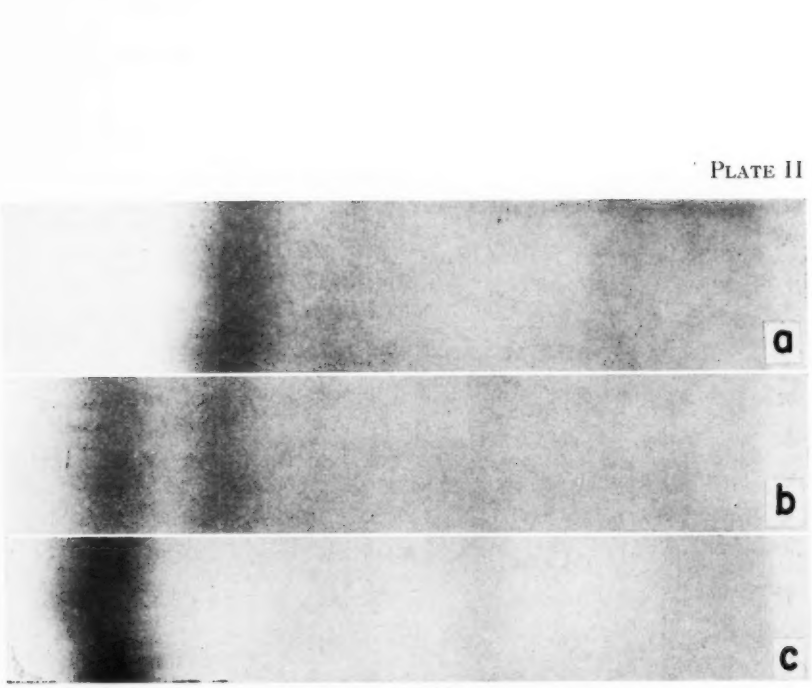

FIG. 3. Electrophoretic pattern of lyophilized serum proteins.
(*a*) Lyophilized serum of normal rat.
(*b*) Mixture of lyophilized sera of normal and Walker tumor-bearing rats.
(*c*) Lyophilized serum of Walker tumor-bearing rat.

which has received the lyophilized serum of the Walker tumor-bearing rat has taken an intense blue color. The skin of the control guinea pig which has received the lyophilized serum of the normal rat has remained unchanged.

The color obtained at the site of the anaphylactic reaction between the antigen and the antibodies is due to the molecules of the Evans blue which escape from the vascular system into the extracellular spaces.

It was of interest to find out which protein fraction of the serum contains this antigenic factor. After the serum of Walker tumor-bearing rats was subjected to electrophoresis according to the method described above, each fraction was assayed for antigenic property by means of the same method. The results are shown in Fig. 2. Of all the fractions studied, only the albumin fraction gives a positive anaphylactic reaction indicating that this fraction has something to do with the antigenic factor that develops in the Walker tumor-bearing organism.

Based upon the assumption that protein such as this serum albumin could hardly acquire an antigenic property without showing a structural modification of some sort, it was interesting to compare its migration rate in paper electrophoresis with that of the normal serum albumin fraction. Therefore, lyophilized sera of Walker tumor-bearing rats, lyophilized sera of normal rats, and a mixture of the two types of lyophilized serum were run on paper electrophoresis under the same experimental conditions.

Figure 3 represents a photograph of the three paper strips, after staining with bromophenol blue. As shown in this figure, the lyophilized serum albumin fraction of tumor-bearing rats has a much faster migration rate than the lyophilized serum albumin fraction of the normal animal.

Attempts were made to relate this higher rate of migration to structural modifications in the serum albumin of Walker tumor-bearing rats. Data obtained from ultracentrifugal analysis and from studies of optical rotation indicate that there is no difference in the two types of albumin, the S uncorrected being 3.89×10^{-13} for the albumin of the normal rat and 3.96×10^{-13} for the albumin of the tumor-bearing rat and the optical rotation being $[\alpha]_D^{20} -39°$ for both types of 1% albumin solution in saline.

There was, however, a very significant difference in the level of polysaccharides in both albumins.

The values obtained for the polysaccharides in the albumins of normal rats and Walker tumor-bearing rats were respectively 70 mg and 300 mg of glucose per 100 g of dry albumin.

Discussion

From the results obtained, it is clear that the Walker tumor induces some changes in the structure of the albumins, as evidenced by the property of antigenicity found and by the different migration rates in electrophoresis.

Ultracentrifugation and optical rotation failed to detect any difference in the two albumins.

However, it has been found that the level of polysaccharides is much higher in the serum albumins of Walker tumor-bearing rats than in the normal serum

albumins. This confirms the data obtained by Shetlar *et al.* (7), who have observed a similar augmentation of polysaccharides.

It has been suggested that polysaccharides have high immunological activity (8). Our observation of a difference in the concentration of polysaccharides in the serum albumin of normal and Walker tumor-bearing rats may account for the differences that we observed in the antigenicity and the different rates of migration of these fractions.

Acknowledgments

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THE INCORPORATION OF FORMATE-C^{14} INTO THE NUCLEIC ACIDS OF RATS WITH REGENERATING LIVER AND NOVIKOFF HEPATOMA[1]

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Abstract

A study was made of the incorporation in vivo of formate-C^{14} into the purines and thymine of regenerating liver and Novikoff hepatoma in the rat, during the period of maximum mitotic activity of these tissues. The effects of these tissues on one another and on certain host tissues were also studied. The maximum mitotic frequency of Novikoff hepatoma was observed on the 4th day of growth following transplantation. This tumor caused a decrease in formate incorporation into the nucleic acid purines and thymine of the host's spleen and intestinal mucosa but had little effect on liver. The results also indicated that the uptake of formate by the RNA adenine of spleen and intestinal mucosa and the DNA thymine of intestinal mucosa was diminished by the presence of regenerating liver. The simultaneous presence of both regenerating liver and Novikoff hepatoma generally lowered the incorporation of formate-C^{14} into the nucleic acids of the host spleen and intestinal mucosa. It was observed further that the utilization of formate by the nucleic acids of Novikoff hepatoma and regenerating rat liver was decreased in animals containing both of these rapidly dividing tissues.

Introduction

In order to study the nucleic acid metabolism of tumor and non-tumor tissue, it was felt that a comparison of a neoplastic and a rapidly growing non-neoplastic tissue would be of interest. For purposes of such a study, Novikoff hepatoma and regenerating rat liver are eminently suitable tissues, since the Novikoff hepatoma is a neoplasm which was originally induced in rat liver and subsequently established in a transplantable form (1), whereas regenerating rat liver is a rapidly growing non-neoplastic tissue. A comparison of these tissues seemed reasonable in view of their common cell origin.

The effects of tumor on the nucleic acid metabolism of several tissues of the host have been demonstrated for a number of tumors. An increase in concentration of nucleic acids in the host tissues of sarcoma-bearing mice has been demonstrated (2–6). Furthermore, Kelly and associates have reported (7–10) an increased incorporation of $P^{32}O_4^{\equiv}$, formate-C^{14}, and glycine-2-C^{14} into the deoxyribonucleic acid (DNA) of host tissues of tumor-bearing animals. Others (11–14) have also observed a stimulation of the incorporation of several preformed purine precursors into the nucleic acids of the host tissues of tumor-bearing animals.

In the experiments to be described a comparison has been made of the incorporation in vivo of formate-C^{14} into the nucleic acid purines and pyrimidines of Novikoff hepatoma and regenerating liver. In addition, the possible effect of one or both of these rapidly growing tissues on the incorporation of

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formate into the nucleic acids of certain tissues of the host was studied. The reciprocal effect on nucleic acid metabolism of regenerating liver and Novikoff hepatoma was also investigated.

Experimental

Groups of male Sprague–Dawley rats (180–200 g each) from the colony at the University of British Columbia were used in all experiments. The Novikoff hepatoma was transplanted by the intraperitoneal injection of 0.5 ml of a 1:5 suspension of minced tumor tissue in physiological saline. Partial hepatectomies were performed using the technique described by Higgins and Anderson (15). Each rat was injected subcutaneously in the dorsal region with 0.3 ml of a solution containing 1 millicurie of sodium formate-C^{14}* dissolved in 5 ml of slightly alkaline water. The quantities of formate-C^{14} injected into each animal, in the experiments to be described, are shown in Table I. This table also summarizes the time relationship of liver regeneration,

TABLE I

Organization of experiments. Animals were sacrified 24 hours after formate-C^{14} injection

Description of animals	Hours of liver regeneration at sacrifice	Day of tumor growth at sacrifice	Number of rats in group	C^{14} injected into each rat, c.p.m.	Formate injected into each rat, mg
1. Partially hepatectomized	40	—	3	3.72×10^7	0.85
2. Tumor-bearing	—	4th	3	3.33×10^7	1.02
	—	6th*	2 or 3 (single)	7.50×10^7	1.03
3. Partially hepatectomized tumor-bearing	40	4th	3	3.33×10^7	1.02
	40	4th	2	4.02×10^7	1.02
4. Normal (intestinal mucosa)	—	—	2	3.94×10^7	1.02
	—	—	2	3.94×10^7	1.02
5. Normal* (spleen, liver)	—	—	2 or 3 (single)	7.50×10^7	1.03

*Data of Zbarsky, Hori, and Findlay (25).

tumor growth, and formate-C^{14} injection, and shows the number of animals used as a group for each experiment. Data for the normal spleen, normal liver, and 6th-day tumor were obtained from the experiments of Zbarsky, Hori, and Findlay (25).

Mitotic Counts

It was felt that a comparison of the formate incorporation into the nucleic acids of Novikoff hepatoma and regenerating liver should be made during the period of maximal growth rate of these tissues. In this way the nucleic acid metabolism of each tissue could be studied under comparable conditions of growth. The maximum mitotic frequency was used as an index of the maximum rate of growth of each tissue.

Information obtained from literature (16, 17) indicated that the period of maximum mitotic frequency of regenerating rat liver occurs approximately

*Purchased from Atomic Energy of Canada Limited or from Merck and Company Limited.

30 hours after partial hepatectomy. The present experiments were designed, therefore, to permit formate incorporation into regenerating liver during the period 16–40 hours after partial hepatectomy. In order to determine the period of maximum mitosis of the Novikoff hepatoma, a group of animals was implanted with tumor suspension. At daily intervals thereafter one animal was sacrificed and a portion of the solid hepatoma excised from the periphery of the tumor mass. Six hours prior to being sacrificed, each rat was injected subcutaneously with colchicine (0.1 mg per 100 g body weight) in order to stop mitosis at the metaphase stage (18). This phase of mitosis was readily identified and allowed a more accurate determination of the mitotic activity. The tissues were prepared for mitotic counts using two techniques.

(*i*) *"Standard" Technique*

The fresh tumor tissue was fixed in formalin, sectioned by a microtome, and the sections were stained with hematoxylin and eosin. Different fields were counted under oil immersion until a total of 500 nuclei had been counted for each tumor.

(*ii*) *"Squash" Technique*

A small portion of the fresh tumor was squashed on a microscope slide to disperse the tissue into a layer one cell in thickness. The "squash" was then stained with acetocarmine and different fields were counted under oil immersion until a total of 1000 nuclei had been counted for each tumor.

Collection of Urine and Expired CO_2

The expired CO_2 and urine were collected using the sealed metabolism cage described by Zbarsky and Wright (19) and Paterson (20).

Isolation and Determination of Purines and Pyrimidines

The animals were killed by exsanguination, under ether anesthesia, and the tissues were quickly removed and frozen. Corresponding tissues from the group of animals were pooled, and the lipids extracted by homogenizing in 95% ethanol, ethanol–ether (3:1), and ether. The fat-free tissues were dried and the nucleic acids extracted as described by Bendich, Russell, and Brown (21). When the wet weight of the tissue was less than 1 g, the nucleic acids were extracted according to the method of Hecht and Potter (22). Ribonucleic acid (RNA) and DNA were separated by the procedure of Tyner, Heidelberger, and LePage (23). The DNA and ribonucleotides were then hydrolyzed with perchloric acid as described by Marshak and Vogel (24). The hydrolyzate was neutralized by the addition of 50% potassium hydroxide, chilled, and the precipitated potassium perchlorate removed by centrifugation. The purines and pyrimidines, contained in the supernatant fluid, were separated by ion exchange chromatography on columns of Dowex-50 (200–400 mesh), H^+ form. The elution was carried out first with 20 to 30 ml of distilled water, followed by 1.5 *N* HCl. The eluate was collected in fractions of 2 to 3 ml using an automatic fraction collector. The optical densities, at 260 mμ, of the fractions were determined using a Beckman model DU spectrophotometer. The fractions containing the individual pyridimines and purines were pooled, evaporated to dryness, and the residues were dissolved in 0.1 *N* HCl and made up to a known

volume. The absorption spectrum of each base was determined using a Beckman DK 2 ratio-recording spectrophotometer, and the concentrations determined from the molar extinction coefficients.

Radioactive Counting Procedures

All radioactive counting was done in a windowless gas-flow counter. Suitable aliquots of the purine and pyrimidine solutions were counted on aluminum planchets covered with lens paper (25). Specific activities of the purines and pyrimidines were expressed as counts per minute per micromole (c.p.m./μmole) and the results for each experiment compared in terms of relative specific activity equal to:

$$\frac{\text{specific activity of purine or pyrimidine (c.p.m./}\mu\text{mole)}}{\text{specific activity of injected formate (c.p.m./}\mu\text{mole)}} \times 10^5.$$

The radioactivity of the expired CO_2 was determined as described by Paterson and Zbarsky (26).

In order to determine the radioactivity in the urine, the sample was made up to volume with alkaline water and the radioactivity determined by counting dried aliquots on aluminum planchets. No correction was made for self-absorption.

Results

Mitotic Counts

On examining the slides prepared by the "squash" technique it was found that the tumor nuclei could be easily detected and counted, since there was adequate dispersion of the tumor cells. Furthermore the fields did not contain tumor cells in which the nuclei were absent. On the other hand, slides prepared by the "standard" technique were difficult to examine because most fields were more than one cell in thickness, the cells were crowded together, and many cells were cut by the microtome so as to exclude their nuclei. It was felt, therefore, that more accurate mitotic counts could be obtained by using the "squash" technique.

An estimation of the mitotic activity of tumor growing during the 1st and 2nd days after transplantation was not possible because insufficient tumor tissue was obtainable at these times. The mitotic index of 7th-day tumor was also not determined because by this time many of the animals had died as a result of the tumor; moreover, the tumor obtained from those animals which did survive was too necrotic and hemorrhagic to permit the determination of the mitotic index.

TABLE II

Mitotic indices of Novikoff hepatoma, as percentage of total nuclei in metaphase

Day after transplantation	% of total nuclei in metaphase	
	"Squash" technique (1000 nuclei counted)	"Standard" technique (500 nuclei counted)
3rd	10	16
4th	10	23
5th	6	8
6th	1	—

From the results of the mitotic counts shown in Table II it is evident that Novikoff hepatoma is most active mitotically during the 3rd and 4th days of intraperitoneal growth. During the following days of growth, the mitotic frequency decreases, so that on the 6th day only about 1% of the nuclei are in mitosis. Although the mitotic index of 3rd-day tumor was similar to that obtained for 4th-day tumor, using the "squash" technique, 4th-day tumor was preferred for the nucleic acid studies because of the larger mass of tumor on that day.

Formate Incorporation into the Nucleic Acids of Tissues of Partially Hepatectomized and Tumor-Bearing Rats

Figures 1 to 3 illustrate the relative specific activities of the nucleic acid purines and DNA thymine obtained from the liver, tumor, spleen, and intestinal mucosa of partially hepatectomized, tumor-bearing, partially hepatectomized tumor-bearing, and normal rats.

The specific activities for uracil and cytosine were also determined in all experiments but are not presented since in most cases they were found to be negligible. Included in Figs. 1 to 3 are data for normal spleen, normal liver, and 6th-day tumor obtained from the experiments of Zbarsky, Hori, and Findlay (25). These data represent the average of results obtained from two or three experiments with single animals.

(*i*) *Partially Hepatectomized Rats*

In order to determine the incorporation of formate-C^{14} into the nucleic acids of regenerating rat liver, each of three rats was partially hepatectomized (15), and injected with formate-C^{14} 16 hours after the operation. Twenty-four hours after the formate injection the animals were sacrificed. The nucleic acid purines and pyrimidines from the regenerating liver, spleen, and intestinal mucosa were analyzed for radioactivity as described. The results obtained for the purines and thymine of 40-hour regenerating liver are shown in Figs. 1 to 3. It is evident that the nucleic acids of regenerating liver incorporate formate-C^{14} to a much greater degree than those of normal liver. The difference is most apparent on comparing the purine data. The data shown indicate that the presence of regenerating liver decreases the incorporation of formate into the RNA adenine of spleen and intestinal mucosa and the DNA thymine of intestinal mucosa.

(*ii*) *Tumor-bearing Rats*

As previously stated, animals bearing 4th-day Novikoff hepatoma were selected for these experiments because of the high mitotic activity observed during the 4th day of intraperitoneal growth. At approximately 50 hours after transplantation, each animal was injected subcutaneously with formate-C^{14}, and sacrificed 24 hours after the formate injection. Therefore each rat contained 4th-day tumor at the time it was sacrificed.

The data from the 4th-day tumor, as indicated in Figs. 1 to 3, show a high degree of incorporation of formate-C^{14} into the nucleic acid purines and thymine. The specific activities of the tumor were greater than those obtained for regenerating liver, normal liver, and normal spleen. The RNA purines of

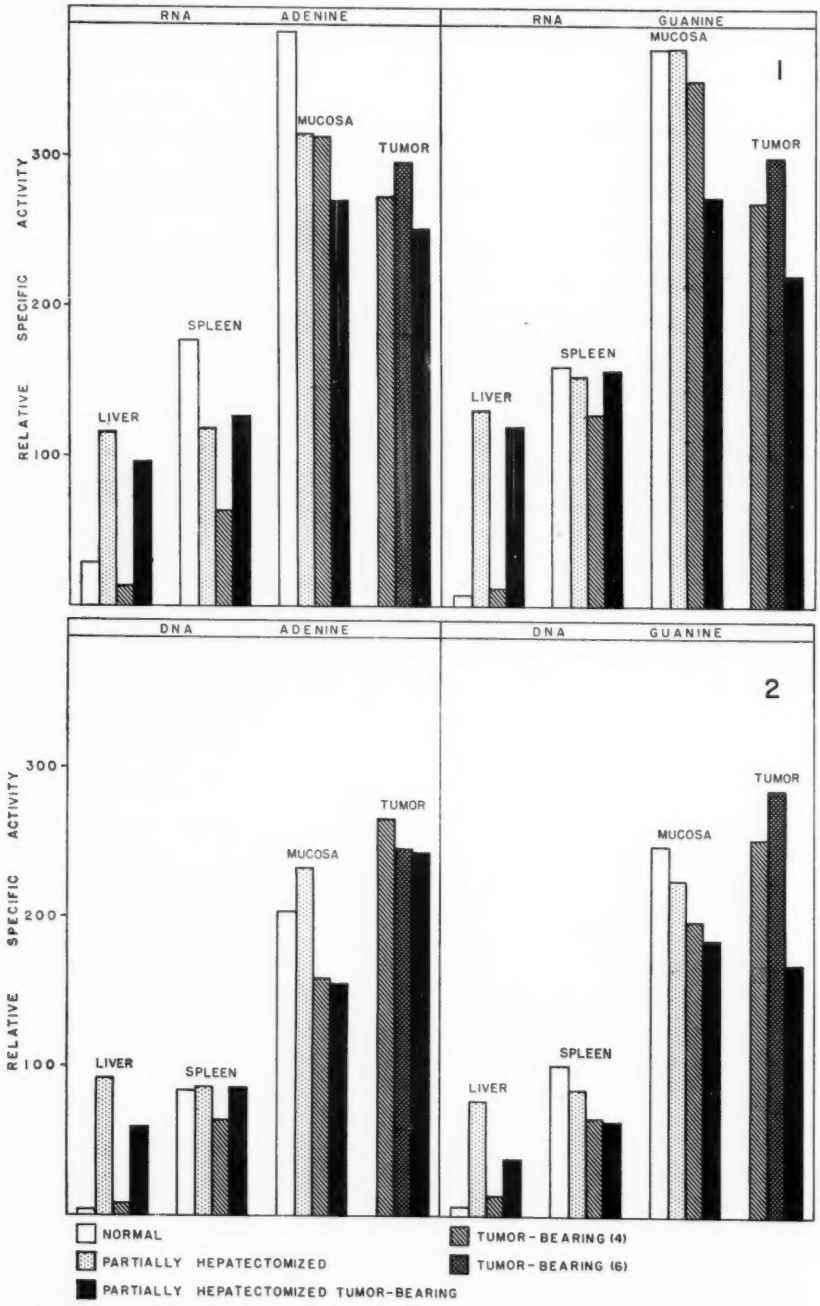

FIG. 1. Relative specific activities of RNA purines from rat tissue 24 hours after the injection of formate-C^{14}. For description of preparation of animals see text.

FIG. 2. Relative specific activities of DNA purines from rat tissue 24 hours after the injection of formate-C^{14}. For description of preparation of animals see text.

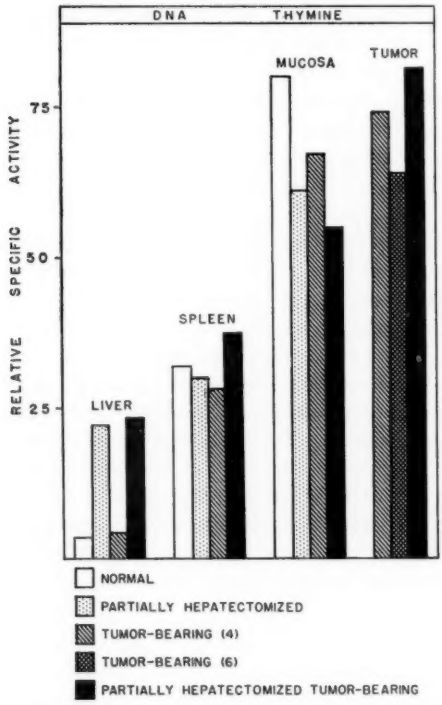

FIG. 3. Relative specific activities of DNA thymine from rat tissue 24 hours after the injection of formate-C^{14}. For description of preparation of animals see text.

tumor had lower specific activities than those of intestinal mucosa whereas the converse relationship was found for the DNA purines. It may be noted that the presence of 4th-day Novikoff heptatoma caused a decrease in the incorporation of formate into the nucleic acid purines and thymine of the host spleen and intestinal mucosa. The greatest decrease is observed in the RNA adenine obtained from the spleens of the 4th-day tumor-bearing animals, compared with the RNA adenine of normal spleen. There was little difference in the specific activities obtained for 4th- and 6th-day tumor.

(*iii*) *Partially Hepatectomized Tumor-bearing Rats*

Since in some cases regenerating liver and 4th-day Novikoff hepatoma had an effect on the incorporation of formate into the nucleic acids of the host tissues of animals containing either one of the above rapidly growing tissues, it was of interest to determine in what manner the simultaneous presence of the two tissues in one animal would affect the formate incorporation into the host tissues. At the same time an opportunity would be provided to study the effect of Novikoff hepatoma and regenerating liver on each other.

With these aims in mind, rats containing both regenerating liver and Novikoff hepatoma were prepared. Partial hepatectomies were performed on

two groups of tumor-bearing rats. The animals were sacrificed 40 hours later, at which time they contained 40-hour regenerating liver as well as 4th-day Novikoff hepatoma. Formate-C^{14} was administered to each animal 24 hours before it was sacrified. The relative specific activities of the nucleic acid bases of the regenerating livers, tumors, spleens, and intestinal mucosa of each group were determined.

The data presented for these experiments in Figs. 1 to 3 represent an average of the results obtained from the two groups. It is apparent that the simultaneous presence of the two rapidly growing tissues (regenerating liver and Novikoff hepatoma) in one animal has generally lowered the incorporation of formate-C^{14} into the nucleic acids of the host spleen and intestinal mucosa, compared with the results shown for these tissues obtained from the normal animals. However, there was a slight increase in the incorporation of formate-C^{14} into the DNA thymine of the spleen of the partially hepatectomized tumor-bearing animals. Furthermore the simultaneous presence of regenerating liver appeared to cause a decrease in the formate uptake by the nucleic acid purines of the tumor while incorporation into the DNA thymine was slightly increased. It was further observed that the presence of Novikoff hepatoma caused a decrease in formate incorporation into the nucleic acid purines of regenerating liver. This decrease was most evident in the case of the DNA purines. However, there was little difference in the results obtained from the DNA thymine of liver.

Excretion of Radioactivity in the Expired CO_2 *and Urine*

The data shown in Table III represent the radioactivity contained in the expired CO_2 expressed as the percentage of total radioactivity injected. Figures given for the partially hepatectomized tumor-bearing animals represent an average of the results obtained from the two groups of animals.

TABLE III

Percentage of injected radioactivity excreted in expired CO_2 by rats given formate-C^{14} subcutaneously

Description of animals	Hours after formate-C^{14} injection						24-hour total
	0–1	1–2	2–3	3–6	6–12	12–24	
Partially hepatectomized	29.4	10.1	3.3	3.3	2.0	1.9	50.0
Tumor-bearing	29.4	8.9	2.0	1.9	1.5	1.5	45.3
Partially hepatectomized tumor-bearing	16.8	12.0	2.8	3.1	2.1	1.8	38.6

It is evident that a considerable fraction of the administered formate was oxidized to CO_2 during the 1st hour, after which the radioactivity of the expired CO_2 decreased. During the 3rd hour the radioactivity decreased to about 1/10th of the initial level, and decreased slowly thereafter. During the 24 hours of each experiment between 38 and 50% or the injected formate-C^{14} was excreted as expired CO_2. The difference between the 50% excreted by the partially hepatectomized animals, and the 38.6% excreted by the partially hepatectomized tumor-bearing animals may not be significant. Others have observed the excretion of 36 to 60% of the administered formate (25, 27) by

normal animals. Therefore it appears that the excretion of formate as CO_2 is not significantly altered by the presence of one or two additional rapidly growing tissues which place a further demand on the "pool" of available formate. The significance of these findings is not understood, although it is possible that the formate "pool" of the rat is large enough that additional demands on the supply of formate would not be manifested in significant alterations in the excretion of formate as CO_2.

Urine samples were collected from each group of animals at periodic intervals following the injection of the formate-C^{14}. Preliminary experiments indicated the presence of a volatile radioactive component(s) in the neutral or acidic urine samples. Therefore, in order to prevent the evolution of this material the urine samples were made alkaline and diluted to known volume with alkaline water. Aliquots of the urine solutions were then counted as described. The nature of the volatile radioactive acidic material(s) was not determined.

The urine counts were not corrected for self-absorption and therefore the results shown in Table IV are approximations of the radioactivity of each urine sample. The data in Table IV represent the radioactivity excreted in the urine, expressed as percentage of the total injected radioactivity. Considerably less of the injected radioactivity is excreted in the urine than in the expired CO_2. The excretion of radioactivity in the urine appears to be greatest during the initial few hours after the formate injection.

TABLE IV

Percentage of injected radioactivity excreted in urine by rats given formate-C^{14} subcutaneously

Description of animals	Hours after formate-C^{14} injection				24-hour total
	0–3	3–6	6–12	12–24	
Partially hepatectomized	3.7	3.3	1.1	1.6	9.7
Tumor-bearing	0.9	0.8	1.0	1.1	3.8
Partially hepatectomized tumor-bearing	2.8	1.2	1.5	1.2	6.7

From Tables III and IV it was calculated that about 45 to 60% of the injected radioactive formate can be accounted for in the CO_2 and urine excreted during the 24 hours immediately following the injection of formate-C^{14}.

Discussion

Several investigators (23, 28–36) have demonstrated a higher incorporation of phosphorus, formate, glycine, orotic acid, and uracil into the nucleic acid fractions of tumor tissue than into those of normal tissues. The results reported herein are for the most part in agreement, for it is evident from these results that formate is more extensively utilized for nucleic acid biosynthesis in 4th- and 6th-day Novikoff hepatoma than it is in normal spleen and liver. However, the high formate incorporation into the hepatoma is not specific to tumor since normal intestinal mucosa exhibits a similar degree of formate uptake. This is in accord with the findings of Heidelberger *et al.* (37), who observed that the degree of conversion of uracil into the nucleic acid pyrimidines of intestinal

mucosa in vivo was similar to that of Flexner–Jobling carcinoma. Therefore a high rate of nucleic acid synthesis or turnover is not unique to neoplastic tissue. It is believed that the differences in the extent of formate incorporation into the nucleic acids of the tissues studied are related to different degrees of growth or cell renewal in these tissues. Since the cells of the intestinal mucosa are continuously being sloughed off and replaced by new cells (38), one would anticipate finding a relatively high level of nucleic acid biosynthesis in this tissue. Furthermore, the higher formate incorporation into the nucleic acids of regenerating liver than into normal resting liver is understandable in view of the greater cell multiplication in the former tissue.

The mitotic counts performed as described indicate that 4th-day hepatoma is a more rapidly dividing tissue than 6th-day hepatoma and might, therefore, be expected to show a higher degree of formate-C^{14} uptake. However, this expectation was not borne out by the experimental findings which showed little difference between the specific activities of 4th- and 6th-day hepatoma.

It is evident, from the results obtained from 4th-day tumor-bearing animals, that the presence of Novikoff hepatoma has some influence on the incorporation of formate into the nucleic acids of spleen and intestinal mucosa of the host. The results indicate that the presence of 4th-day tumor caused a decrease in formate incorporation into the nucleic acid purines and thymine of the host spleen and intestinal mucosa. However, 4th-day tumor had little effect on the formate uptake into the nucleic acids of liver. In agreement are the findings of LePage and Heidelberger (29), who found no difference in glycine incorporation into the nucleic acids of normal liver as compared with livers of rats bearing Flexner–Jobling carcinoma. In contrast others have reported (7–14, 25) that several nucleic acid precursors were utilized to a greater degree by the nucleic acids of host tissues of tumor-bearing animals than those of tissues of normal animals.

No evidence was obtained to support the concept of the release of a "growth-promoting" agent from the regenerating liver of rats as proposed by others (39). On the contrary, the results reported herein indicated that the *de novo* biosynthesis of RNA adenine of spleen and intestinal mucosa, and the DNA thymine of intestinal mucosa was decreased by the presence of regenerating liver in the animal. Furthermore, Novikoff hepatoma and regenerating liver appeared to cause a decrease in the uptake of formate into the nucleic acid purines of one another in partially hepatectomized tumor-bearing rats. However, these findings do not agree with the growth experiments of Paschkis *et al.* (39). Balis and associates (40) have found a stimulation in the uptake of several preformed precursors into the purines of tissues of partially hepatectomized hamsters and an increased incorporation of adenine into the tumor of partially hepatectomized hamsters bearing a human tumor. On the other hand, the utilization of glycine by the human tumor in the partially hepatectomized hamster was reduced. It appears, therefore, that the presence of regenerating liver stimulates the incorporation of preformed purines into the nucleic acids of tumor but depresses the *de novo* pathway. However, Balis and associates (40) concluded from preliminary experiments with other tumors and

animals that the effects of regenerating liver on tumor were not consistent. Using partially hepatectomized rats bearing subcutaneous transplants of Flexner–Jobling carcinoma, LePage and Heidelberger (29) found that tumor and regenerating liver did not influence the incorporation of glycine into the nucleic acids of one another.

It appears that under the conditions of the present experiments the combination of regenerating liver and Novikoff hepatoma in one rat alters the nucleic acid metabolism of the host's spleen and intestinal mucosa. There was a decrease in the relative specific activities of most of the nucleic acid purines and thymine from the spleen and intestinal mucosa of the partially hepatectomized tumor-bearing rats. In contrast, Balis and co-workers (40) found no effect on the nucleic acid metabolism of host tissues in partially hepatectomized hamsters bearing human tumors and observed that the tumor had no effect on the nucleic acid metabolism of regenerating liver. The results of the present investigation disagree somewhat, for it was observed that the presence of Novikoff hepatoma decreased the incorporation of formate into the nucleic acid purines of regenerating liver in partially hepatectomized tumor-bearing rats.

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ON THE DEVELOPMENT OF INCREASED TRYPTOPHAN SYNTHETASE ENZYME ACTIVITY BY CELL-FREE EXTRACTS OF NEUROSPORA CRASSA[1]

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Abstract

A cell-free extract from conidia of *Neurospora crassa* developed increased tryptophan synthetase activity when incubated with a mixture of amino acids, a source of energy, and inorganic salts. Fifteen individual acids were required for development of maximal increases of enzymic activity. Either hexose diphosphate or adenosine triphosphate could serve as energy source. Diphosphopyridine nucleotide, polyvinyl acetate, and magnesium and calcium ions were required. The addition of ribonuclease enzyme, ethionine, fluorophenylalanine, thienylalanine, or various antibiotics prevented development of increased tryptophan synthetase activity. The increase of activity was accompanied by a net synthesis of protein.

Introduction

Many subcellular systems have been shown to catalyze the formation of new protein (see, for example, refs. 1–11) or an incorporation of radioactive amino acid into the protein fraction of the system (for example, refs. 12–19). Of these systems, a number have been reported to form identifiable, specific proteins (1–9). However, only two of this latter group of systems are derived from an organism which is amenable to the fine resolution afforded by the application of genetic techniques. Further, both of these make use of "protoplast" preparations of *Escherichia coli*, which are capable of forming inducible β-galactosidase enzyme (4, 20) and also proteins of bacteriophage T_2 (see, for example, ref. 1).

Additional study of the phenomenon of transformation recently reported for strains of *Bacillus subtilis* (21) will probably permit the application of genetic techniques to some of the other systems currently in use (see, for example, refs. 4, 9). Nevertheless, it seems desirable to develop other subcellular systems from organisms which have been the subject of intensive genetic study.

Accordingly, we have developed from *Neurospora crassa* subcellular systems which are capable of synthesizing protein and, in particular, forming additional tryptophan synthetase (desmolase) enzyme activity. This enzyme has been considerably purified and its enzymic properties characterized (22, 23). The enzyme is antigenic and neutralizing antisera can be prepared (24). Further, extensive genetic studies have provided a series of pseudoallelic mutants all characterized by the inability to form active tryptophan synthetase enzyme (25, 26, 27, 28).

The present communication reports studies made with preparations obtained from conidia. The information obtained has permitted development of a system from growing mycelium, which will be reported upon in a subsequent communication.

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Methods

(*a*) *Organism and Stock Cultures*

All experiments were made with extracts from washed conidia of the 74A strain of *Neurospora crassa*.

Stock cultures were grown on agar slants of ca. 10-ml volumes of a minimal medium solidified with 2% agar in 16×150-mm test tubes. The medium contained KNa tartrate 5.0 g, $NaNO_3$ 3.0 g, KH_2PO_4 3.0 g, $MgSO_4.7H_2O$ 0.5 g, K_2SO_4 0.3 g, NaCl 0.1 g, $CaCl_2$ 0.1 g, biotin 0.5 μg, trace elements (29), and sucrose 20 g per liter. Tubes were inoculated with 0.05-ml volumes of suspension prepared as below, incubated at 30° C for 4 days, and transferred to room temperature (ca. 20° C) to induce conidiation.

(*b*) *Preparation of Large Batches of Conidia*

Experimental batches of conidia were grown on 500-ml volumes of medium in 2-liter Fernbach flasks. The conidia of one stock slant were suspended in 10 ml of sterile distilled water by rubbing gently with a glass rod, and a 3-ml volume of the resulting suspension was used as the inoculum for one Fernbach flask. Several flasks were inoculated at the same time and they were incubated at 30° C for 4 days. The flasks were then transferred to room temperature to induce conidiation, and the cotton stoppers were replaced by a single ply of facial tissue to increase aeration. (The latter procedure resulted in increases of conidial yields by ca. 50% and rarely permitted visible contamination of the culture.) After further incubation at room temperature (ca. 20° C, well-lit exposure) for 3 to 5 days, the conidia were suspended in ca. 250 ml of distilled water per flask by gentle rubbing with a glass rod. The suspension was filtered through gauze to remove fragments of mycelium, and the conidia were harvested by vacuum filtration through a Whatman No. 4 filter paper. This procedure yielded up to 5 g wet weight of conidia per Fernbach flask, and for most experiments three flasks were harvested.

The cake of conidia was weighed, resuspended in ca. 500 ml of distilled water with 1½ parts (w/w) of powdered Pyrex glass (200 mesh), and the slurry was refiltered. The suspension was stirred occasionally during the filtration in order to obtain a good admixture of glass and conidia in the resulting cake, which was sucked as dry as possible. The cake was crumbled and stored overnight at 4° C.

(*c*) *Preparation of Extracts*

Portions of the chilled cake of 6 to 10 g wet weight were lightly ground (see below) in previously chilled mortars with an additional 2 g of powdered glass and 0.4 g sucrose per portion, and rapidly suspended in from 10 to 20 ml per wet weight of conidia of cold *M*/20 sodium potassium phosphate buffer (pH 6.5) containing 20% sucrose.

This grinding procedure is extremely critical and, even with our extensive experience, approximately 10% of the extracts prepared recently have not developed increased tryptophan synthetase activity on subsequent incubation. It is essential that the grinding be both light and brief. In our experience, it is advisable to cease as soon as the preparation appears "moist", as judged by a change in hue of the preparation from very pale to dark orange.

The slurry in sucrose–phosphate buffer was centrifuged at 200 *g* for 10 minutes at 2° C to remove the glass, unbroken conidia, and gross cell debris, and then filtered through glass wool to remove the "raft" of small air bubbles which usually formed at the surface of the tubes. The supernatant fraction obtained at this juncture should be pale orange in color. Extracts of lighter hue indicate inadequate rupture of the conidia, whereas extracts of a dark orange color fail to develop either increased tryptophan synthetase activity or an increased content of total protein N.

The foregoing supernatant fraction was recentrifuged at 20,000 *g* for 20 minutes at 2° C to remove the mitochondria, and the resulting supernatant fraction was used as the experimental extract. In some experiments the extract was supplemented with $CaCl_2$ and $MgSO_4$, as indicated, and separated into "soluble" and "particulate" fractions by centrifuging at 2° C for 1 hour in a Spinco model L preparative ultracentrifuge at an average force of 105,000 *g*. The "particulate" fraction was resuspended in 1/12 of the original volume of cold *M*/20 sodium potassium phosphate buffer (pH 6.5) containing 20% sucrose.

(*d*) *Reaction Mixtures*

Unless otherwise stated, 14-ml aliquots of extract, in 125-ml Erlenmeyer flasks, were diluted with 14 ml of reagent solution plus test materials, and incubated aerobically at 30° C for 1 hour. The composition of the reagent solution added was modified progressively throughout the work and the composition for each experiment is referred to the solutions listed in Table I.

TABLE I

	Reagent solution, No.			
	1 Original synthetic	2 Final synthetic	3 Original complex	4 Final complex
Mixed amino acids,* each	8	8	—	—
L-Arginine HCl	8	—	—	—
L-Cysteine	6.4	6.4	6.4	6.4
Glycine	8	—	—	—
L-Serine	8	—	—	—
L-Threonine	8	3.2	—	—
L-Tryptophan	3.2	1.6	—	—
L-Tyrosine	3.2	3.2	—	—
L-Valine	8	3.2	—	—
Casein hydrolyzate	—	—	560	1120
Hexose diphosphate (Mg salt)	50	50	50	50
Diphosphopyridine nucleotide	4	4	4	4
$MgSO_4.7H_2O$	98.6	493	493	493
$CaCl_2$	0.4	0.4	0.4	0.4
Polyvinyl acetate	—	—	—	42

NOTE: Compositions of the reagent solutions in milligrams per 14 ml (final) of distilled water. The final complex solution (No. 4) contained *M*/56 sodium potassium phosphate buffer (pH 6.5).

*The mixed amino acids consisted of the L-isomers of alanine, aspartic acid, glutamic acid, histidine HCl, isoleucine, leucine, lysine HCl, methionine, phenylalanine, and proline.

(*e*) *Isolation of Tryptophan Synthetase Enzyme*

Procedure A.—In early experiments the tryptophan synthetase enzyme was precipitated from 20 ml of reaction mixture by adding 50 ml of cold saturated

$(NH_4)_2SO_4$ (pH 7.8). The precipitate was spun down after standing for 1 hour at 4° and redissolved in 5 ml of *M*/10 sodium potassium phosphate (pH 7.8).

Procedure B.—When Suskind and Kurek reported the presence of a complex inhibitor of tryptophan synthetase activity in extracts of *N. crassa* (28) a modification of their fractionation was adopted. Samples of 24 ml of reaction mixture were supplemented with a final concentration of *M*/1200 reduced glutathione, and adjusted to a pH of 6.8 to 6.9 with 5% NaOH. Then 3.4 ml of 1.5% protamine sulphate was added, and the sample was centrifuged at 5000 *g* for 10 minutes at 2° C. The supernatant was adjusted to a pH of 5.8 with *M*/5 acetic acid, and solid $(NH_4)_2SO_4$ was added to a final concentration of 25%. After standing for 1 hour at 4° C the precipitate was spun down at 20,000 *g* for 15 minutes at 2° C and redissolved in 3 ml of *M*/10 sodium potassium phosphate buffer (pH 7.8).

(*f*) *Assay of Tryptophan Synthetase Activity*

Tryptophan synthetase activity was determined by a modification of the method of Yanofsky (22). Each tube contained 0.2 ml *M*/2 sodium potassium phosphate buffer (pH 7.8), 0.08 ml (later experiments) or 0.12 ml (early experiments) *M*/200 indole, 0.5 ml enzyme preparation, 0.4 ml of 1 mg/ml pyridoxal phosphate solution, 0.1 ml 1% serum albumin (fraction V), 0.02 ml *M*/20 reduced glutathione, and 0.6 ml *M*/5 DL-serine. For each enzyme preparation, the activity was determined at 30° C over 20-minute and 40-minute periods. The reaction was stopped by adding 0.5 ml 5% NaOH, and the residual indole was determined according to Yanofsky (22). Control tubes received NaOH prior to incubation.

The unit of tryptophan synthetase activity is defined as the amount of enzyme which catalyzes the disappearance of 0.1 μmole of indole per hour at 30° C. Enzyme activities are expressed as units of enzyme per milliliter of conidial extract.

(*g*) *Determination of Total Protein N*

For the determinations of total protein content of the reaction mixtures, 20-ml samples were precipitated with 20 ml of cold 20% trichloroacetic acid and centrifuged at 5000 *g* for 10 minutes. The precipitate was transferred to a smaller centrifuge tube by suspension in 5 ml of 2% NaOH, reprecipitated with 6 ml of 20% trichloroacetic acid, and recentrifuged. The final reaction mixture developed contained polyvinyl acetate and, where necessary, the sample was freed of the polymer by resuspension in 5 ml of 2% NaOH and recentrifuging at 5000 *g*, followed by precipitation with 6 ml of 20% trichloroacetic acid. The precipitates were subjected to a further two cycles of solution in 1% NaOH and reprecipitation with 10% (final) trichloroacetic acid.

The precipitates were then successively extracted with 10- to 12-ml volumes of hot absolute ethanol (90° C for 15 minutes), ethanol–chloroform (3:1), acetone, ethanol – petroleum ether (3:1), and petroleum ether (b.p. 65–110° C). The treated precipitates were then extracted once with 3 ml of hot 5% trichloroacetic acid (15 minutes at 90° C) and once with 1 ml of 5% trichloroacetic acid at room temperature. The final precipitate was dissolved in 2 to 4 ml of

1% NaOH and used for assays of total protein N by standard micro-Kjeldahl distillation techniques, using Nessler's reagent.

(*h*) *Materials*

L-Alanine, L-cysteine hydrochloride, L-glutamine, L-isoleucine, L-methionine, L-proline, L-serine, DL-serine, L-threonine, L-tryptophan, L-valine, L-ethionine, β-2-thienylalanine, reduced glutathione, serum albumin fraction V, deoxyribonuclease, protamine sulphate, penicillin G, streptomycin sulphate, dihydrostreptomycin sulphate, neomycin sulphate, bacitracin, usnic acid, pyridoxal phosphate, diphosphopyridine nucleotide, and crystalline vitamin B_{12} were purchased from the Nutritional Biochemicals Corporation.

L-Aspartic acid, L-asparagine, L-arginine monohydrochloride, L-glutamic acid, glycine, L-leucine, L-lysine monohydrochloride, L-phenylalanine, L-tyrosine, DL-*p*-fluorophenylalanine, 8-azaguanine, 2-thiouracil, and hexose diphosphate magnesium salt were obtained from Mann Laboratories, Inc. L-Isoleucine was purchased from the California Corporation for Biochemical Research. Adenosine triphosphate and ribonuclease were supplied by the Sigma Chemical Company. The casein hydrolyzate used was "Difco, Bacto Casamino Acids", and coenzyme A was obtained from the Pabst Laboratories.

Neomycin sulphate, tetracycline hydrochloride, and polymyxin B were gifts of Pfizer of Canada Corporation, erythrocin was given by Abbott Laboratories Ltd., and chloramphenicol was given by Parke Davis and Co. Ltd.

The polyvinyl acetate preparation was DuPont "Elvacet" grade 81-900, a gift of DuPont of Canada Ltd.

Results

(*a*) *The Assay of Tryptophan Synthetase Activity*

Pyridoxal phosphate is an essential coenzyme for the tryptophan synthetase enzyme of *N. crassa* (22). Therefore, it seemed possible that any increase of apparent tryptophan synthetase activity shown by the assay method of Yanofsky (22) could be attributed either to a process involving a reduction in the concentration of pyridoxal phosphate required for optimal activity, or to a reduction in the rate of destruction of the cofactor by our crude enzyme preparations. Accordingly, at various stages throughout the development of our system, we examined the effect of variation of the pyridoxal phosphate concentration upon the apparent tryptophan synthetase activity of preparations precipitated from the reaction mixture before and after incubation. At all stages, and using either precipitation procedure, the highest activities were assayed at concentrations of pyridoxal phosphate of 210 μg/ml, which is 10 times greater than that used in Yanofsky's method (22). Halving the concentration of pyridoxal phosphate caused a very marked reduction in the apparent tryptophan synthetase activities of our preparations (Table II).

Pyridoxal phosphate could not be replaced by pyridoxal hydrochloride, pyridoxamine hydrochloride, or pyridoxine hydrochloride. There was no disappearance of indole in the absence of serine, or with enzyme preparations heated for 5 minutes in a boiling-water bath.

TABLE II

Influence of pyridoxal phosphate concentration on apparent tryptophan synthetase activity

Expt. No.	Precipitation procedure (see Methods)	Apparent tryptophan synthetase activity, units/ml of original extract	
		Concn. of pyridoxal phosphate: 210 μg/ml	105 μg/ml
1	A	0.35	0.10
2	A	0.80	0.53
3	A	0.71	0.49
	B	0.85	0.41
4	B	0.66	0.33
5	B	0.43	0.27

NOTE: In all these experiments enzyme was precipitated from a reaction mixture consisting of equal parts of conidial extract and reagent solution 3 after aerobic incubation for 1 hour at 30° C.

Apparent tryptophan synthetase activities were directly proportional to the concentration of enzyme preparation over the relatively narrow range of activities spanned in this study.

(*b*) *Isolation of the Apparent Tryptophan Synthetase Enzyme from the Reaction Mixture*

The tryptophan synthetase reaction is inhibited by amino acids (22). Indeed, no tryptophan synthetase activity whatsoever could be detected in our complex reaction mixtures, and it was necessary to separate the enzyme from the small molecules present in the experimental system. In early experiments this was accomplished by precipitating the enzyme with ammonium sulphate at approximately 70% saturation (pH 7.8) (procedure A, Methods). As all the enzymic activity can be precipitated when the mixture is brought to 45% saturation with ammonium sulphate, it seemed unlikely that any apparent increase of tryptophan synthetase activity resulting from incubation of the reaction mixture could be attributed to the incomplete precipitation of active enzyme from the unincubated mixture. However, it did seem possible that the increased activity might be due to metabolic destruction of some inhibitor which coprecipitated with the enzyme under our experimental conditions.

As a first examination of this possibility, "recovery" experiments were performed. Equal parts of unincubated and incubated reaction mixture were pooled to give an artificial sample, which was processed in parallel with the customary experimental samples. The apparent tryptophan synthetase activity found for the artificial sample was equal to the mean value of the activities obtained with the two experimental samples (Table III). It therefore seemed unlikely that the observed increases of enzymic activity were due to destruction of an inhibitor of the enzyme, particularly in view of the requirements for development of the increased activity reported later.

However, when Suskind and Kurek (28) reported that some of the tryptophan synthetase enzyme in extracts of wild-type *N. crassa* was complexed with firmly bound inhibitor in an enzymically inactive form, further investigation was undertaken.

TABLE III

Recovery experiment

Expt. No.	Tryptophan synthetase activities, units/ml of original extract			
	Unincubated sample* 1	Incubated sample 2	Mean of 1 and 2	Artificial sample
1	0.70	1.05	0.88	0.88
2	0.42	0.85	0.64	0.63

NOTE: The activity obtained from a mixture of unincubated and incubated reaction mixtures was compared with the expected mean value (see text). Reagent mixture 1. Precipitation procedure A.
*This sample was stored at 4° C during the period of incubation. The treatment did not affect the apparent tryptophan synthetase activity of the sample.

Samples of both unincubated and incubated reaction mixtures were precipitated in parallel by the procedure (A) which had been adopted up to this point, and by a modification of the procedure used by Suskind and Kurek to separate the enzyme from the inhibitor (procedure B, see Methods). When the resulting enzyme fractions were assayed it was found that our preparations did apparently contain appreciable amounts of the firmly bound inhibitor. However, the amount of enzyme apparently present in inactive form did not change during the course of the incubation (Table IV). Consequently, the increase of apparent tryptophan synthetase activity developed in our system could not be attributed to destruction of the firmly bound inhibitor.

TABLE IV

Firmly bound inhibitor and the increase of tryptophan synthetase activity

Expt. No.	Tryptophan synthetase activity, units/ml original extract					
	Unincubated sample		Incubated sample		Differences	
	Pptn. A (1)	Pptn. B (2)	Pptn. A (3)	Pptn. B (4)	(2−1)	(4−3)
1	0.39	0.54	0.65	0.80	0.15	0.15
2	0.49	0.60	0.71	0.84	0.11	0.13
3	0.33	0.52	0.49	0.66	0.19	0.17
4	0.29	0.36	0.36	0.43	0.07	0.07
5	0.37	0.43	0.49	0.54	0.06	0.05

NOTE: All experiments were made with reagent solution 3 (see Methods). For details of the experiments see the text.

(*c*) *Conditions for Development of Increased Tryptophan Synthetase Activity*

One of the greatest difficulties encountered in the early stages of this work was in judging the amount of conidial extract likely to develop an increase in tryptophan synthetase activity. Indeed the range of concentrations of conidial material which could be used was extremely narrow. Our extensive data suggested that the extract contained proteolytic enzymes, and that a critical balance must be struck between the amount of preformed protein added to the system and the concentration of amino acids.

The increase in tryptophan synthetase activity appeared to be complete within 1 hour of incubation, using either synthetic reagent solution 1 or complex solution 4. The activity developed was stable during incubation for a further hour (Fig. 1).

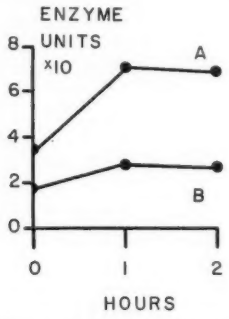

FIG. 1. Time course of development of tryptophan synthetase activity.
Curve A: Reaction mixture 1 (Table I). Precipitation procedure A (see Methods).
Curve B: Reaction mixture 4 (Table I). Precipitation procedure B (see Methods).
Ordinate: Tryptophan synthetase activity per milliliter original extract. Extract was incubated aerobically at 30° C.

The increase of tryptophan synthetase activity developed was as great under anaerobic conditions as in aerobic reaction mixtures.

All of the reaction mixtures contained conidial extract, amino acid mixture, energy source, diphosphopyridine nucleotide, magnesium and calcium ions. In addition, the final system contained polyvinyl acetate (tested as a substitute for polyvinyl sulphate, an inhibitor of ribonuclease enzyme (6)). All of these components were required for the development of maximal increases of tryptophan synthetase activity. The constituents of the conidial extract and the amino acid mixture are considered separately in later sections. Pertinent data for the other components of the reaction mixture are given in Tables V–VII, and the optimal concentrations of these components were as given in Table I.

Three observations warrant some comment. For convenience and economy, hexose diphosphate was normally used as the source of energy. It could be replaced by adenosine triphosphate, but a combination of the two energy sources was no more effective than either alone (Table VI).

The required concentration of magnesium sulphate was very high (*M*/14 final) in all but the first of the reaction mixtures (Table I). Attempts were made to replace a portion of the magnesium requirement by manganous chloride, zinc sulphate, and by a mixture of trace elements (29), but without success.

TABLE V

Requirements for calcium ions and diphosphopyridine nucleotide

Expt. No.	Tryptophan synthetase activities, units/ml original extract			
	Unincubated sample	Complete system*	System without Ca^{++}	System without diphosphopyridine nucleotide
1	0.71	1.06	0.88	—
2	0.70	0.86	0.70	—
3	0.52	0.78	—	0.69
4	0.64	0.94	—	0.76

*The complete system consisted of extract and reagent solution 1. Incubation for 1 hour at 30° C aerobically. Enzyme assays were made on preparations precipitated by procedure A.

TABLE VI

Requirement for energy source

Expt. No.	Tryptophan synthetase activities, units/ml original extract				
	Unincubated control	No phosphorylated energy source	Complete system with HDP*	Complete system with ATP†	Complete system with HDP and ATP‡
1	0.81	0.45	1.08	—	—
2	0.54	0.50	1.08	—	—
3	0.28	—	0.55	0.50	0.50
4	0.15	—	0.25	0.23	0.30
5	0.38	—	0.68	—	0.68

*For experiments 1 and 2 the complete system consisted of extract and reagent solution 1. For experiments 3–5 the system consisted of extract and reagent solution 2.
†Reagent solution 2 containing 25 mg disodium ATP instead of the hexose diphosphate.
‡Reagent solution 2 supplemented with 25 mg disodium ATP. Incubation 1 hour at 30° C aerobically. Enzyme assays were made on preparations precipitated by procedure A.

TABLE VII

Requirement for magnesium ions and polyvinyl acetate

Expt. No.	Tryptophan synthetase activities, units/ml original extract			
	Unincubated control	Complete system*	With reduced $[Mg^{++}]$†	Complete system less polyvinyl acetate
1	0.25	0.37	0.27	—
2	0.19	0.27	0.15	—
3	0.63	0.79	—	0.63
4	0.18	0.31	—	0.18
5	0.38	0.46	—	0.38
6	0.51	0.97	—	0.58

*The complete system consisted of extract and reagent solution 4.
†Reagent solution 4 containing 20% of the Mg^{++} concentration given in Table I. Incubation aerobically for 1 hour at 30° C. Enzyme activities were assayed with preparations precipitated by procedure B.

Perhaps the most surprising finding was that on doubling the concentration of casein hydrolyzate in reagent solution 3 (Table I) we introduced a requirement for polyvinyl acetate. Up to this point we had regularly obtained increases of 50 to 100% in the tryptophan synthetase activity during incubation with reagent solutions 1–3. Nevertheless, as shown in Table VII, there was no significant increase of enzyme activity during incubation with reagent solution 4 lacking the synthetic polymer.

The rise in tryptophan synthetase activity was not increased by supplements of various mixtures of nucleic acid derivatives (see, for example, refs. 2, 13), by coenzyme A (5), vitamin B_{12} (30), or by pyridoxal phosphate.

(*d*) *Requirements for Amino Acids*

Our first reagent solution (number 1) was based on the reaction system of Gale and Folkes (2), and contained 18 amino acids. Experiments were made to determine which of these amino acids were required to increase maximally the tryptophan synthetase activity, and to obtain a rough indication of the optimal concentration needed. The test acid was omitted from either reagent solution 1 (Table I), or a modification of this solution made after earlier tests of another amino acid. Results obtained in this manner are summarized in

Table VIII. Although, for some of the acids, an absolute requirement was found in most experiments of a series, in other experiments no supplement was

TABLE VIII

Requirements for amino acids

(*a*) Acids required:
Alanine (4/6), aspartic acid (4/5), cysteine (4/4), glutamic acid (4/6), histidine (4/6), isoleucine (4/6), leucine (4/4), lysine (3/3), methionine (3/3), phenylalanine (3/3), proline (3/3), threonine (3/4), tryptophan (7/9), tyrosine (4/4), valine (3/5)

(*b*) Acids and amides not required:
Glycine (0/4), asparagine (0/2), glutamine (0/2)

(*c*) Inhibitory acids:
Arginine (0/4), serine (0/3)

NOTE: All tests were made with L-isomers; for details see the text.

required, presumably because the crude extract contained a sufficient quantity of the test acid. The figures given in parentheses show the proportion of experiments in which the acid was required.

Acids which were not required were omitted in the further development of the reaction mixture, and wherever an optimal concentration was established the composition of the reagent solution was modified accordingly. This procedure resulted in the development of reagent solution 2 (Table I).

The only amino acid of special interest was L-tryptophan, which has been shown to inhibit formation of tryptophan synthetase enzyme by growing mycelium of *N. crassa* (22). Most of our extracts (seven of nine) did show a requirement for L-tryptophan. Nevertheless, the extent of the rise in tryptophan synthetase activity was very markedly influenced by small changes of tryptophan concentration (Table IX).

TABLE IX

Influence of tryptophan on development of tryptophan synthetase activity

Expt. No.	Tryptophan synthetase activities, units/ml original extract					
	Unincubated control	Tryptophan, μg/ml				
		0	29	57*	114†	286
1	0.31	0.23	0.25	0.73	0.67	0.17
2	0.37	0.39	0.53	0.73	0.39	0.25
3	0.22	0.16	0.27	0.41	0.22	0.22

NOTE: Extract was incubated aerobically at 30° C for 1 hour with a modified reagent solution 1, lacking arginine, glycine, and serine, and containing varying amounts of tryptophan. Precipitation procedure A.
*Tryptophan concentration in reagent solution 2.
†Tryptophan concentration in reagent solution 1.

Apart from the requirement for cysteine, the synthetic amino acid mixture could be completely replaced by 2% (final) casein hydrolyzate (solution 3). In the presence of polyvinyl acetate, somewhat larger increases of tryptophan synthetase activity were obtained when the concentration of casein hydrolyzate was raised to 4% (solution 4, Table I).

(*e*) *Requirement for "Particulate" and "Soluble" Fractions of the Extract*

On standing at 4° C for 1 hour, extracts prepared by the standard procedure given under Methods lost the ability to develop increased enzymic activity.

However, when they were stabilized by adding 2 ml of 0.1% $CaCl_2$ and 1 ml of $M/1$ $MgSO_4$ per 60 ml of extract they could be separated into "particulate" and "soluble" fractions by ultracentrifugation at an average force of 105,000 *g* for 1 hour. Both components were required for development of increased tryptophan synthetase activity. In the range tested, the extent of the increase was roughly proportional to the concentration of the "particulate" material when tested with a constant amount of "soluble" phase. Similarly, the amount of new enzyme activity formed was roughly proportional to the concentration of "soluble" phase when using a constant amount of "particulate" material (Table X).

TABLE X

Participation of "particulate" and "soluble" fractions in the development of increased enzymic activity

	Tryptophan synthetase activities, units/ml original extract				
				Complete system† plus:	
Expt. No.	Unincubated controls* A	Unincubated controls* B	C nil	D "particulate" fraction	E "soluble" fraction
1	0.24	0.48	0.34	0.40	0.67
2	0.11	0.23	0.18	0.23	0.36

NOTE: Extract was separated into "particulate" and "soluble" fractions by ultracentrifugation (see text).
*Control A corresponded to the complete system (flask C), and control B corresponded to the reaction system in flask E. Incubation for 1 hour at 30° C aerobically. Precipitation procedure B.
†The complete system (flask C) consisted of "particulate" and "soluble" fractions recombined in the original proportions plus reagent solution 4. The final concentration of reconstituted extract was equivalent to half that given under Methods. In flask D the concentration of "particulate" fraction was doubled, and in flask E the concentration of "soluble" fraction was doubled.

(f) Action of Nuclease Enzymes, Metabolite Analogues, and Other Inhibitors

Addition of 20 μg/ml of crystalline ribonuclease totally inhibited the development of increased tryptophan synthetase activity, both in synthetic (No. 1) and complex (No. 4) reaction mixtures.

Effects of 20 μg/ml of crystalline deoxyribonuclease were more complex. However, the addition of deoxyribonuclease to the final complex medium (No. 4) invariably caused total inhibition of the rise in tryptophan synthetase activity.

There was no increase in tryptophan synthetase activity within 1 hour in the presence of the following substances at the concentrations given: 1.8×10^{-2} *M* sodium azide, 2.1×10^{-5} *M* 2,4-dinitrophenol, 10^{-3} *M* ethionine, 10^{-3} *M* *p*-fluorophenylalanine, 10^{-3} *M* β-2-thienylalanine, 41 μg/ml chloramphenicol, 20 μg/ml penicillin, 300 μg/ml streptomycin sulphate, 100 μg/ml dihydrostreptomycin sulphate, 0.5 μg/ml tetracycline hydrochloride, 100 μg/ml erythrocin, 50 μg/ml polymixin B, 50 μg/ml neomycin sulphate, 50 μg/ml bacitracin, and 100 μg/ml usnic acid. Azide and dinitrophenol were tested in reagent solution 1, using precipitation procedure A. All other substances were tested in reagent solution 2, using precipitation procedure A. In addition, ethionine, fluorophenylalanine, thienylalanine, chloramphenicol, and penicillin were retested in solution 4, using precipitation procedure B. Control experiments showed that none of these substances were coprecipitated with the

enzyme in amounts sufficient to cause inhibition of the enzymic activity during assay.

Azaguanine (50 μg/ml) and thiouracil (100 μg/ml) were without effect on the development of tryptophan synthetase activity.

(*g*) *Increased Enzymic Activity and the Net Synthesis of Protein*

Development of increased enzymic activity did not, per se, seem an adequate criterion of protein synthesis. Accordingly, experiments were made to determine whether there was a parallel increase in total protein content of the system. Results obtained under our final experimental conditions are shown in Table XI. As our crude extracts could catalyze the degradation of protein,

TABLE XI

Parallel increases in enzymic activity and in total protein

Expt. No.	Tryptophan synthetase activities, units/ml original extract		Protein N content, μg/ml original extract	
	Unincubated	Incubated	Unincubated	Incubated
1	0.34	0.46	26	32
2	0.18	0.27	38	47
3	0.20	0.34	33	38

NOTE: Extract was incubated aerobically at 30° C for 1 hour with reagent solution 4. Enzymic activities were assayed with preparations obtained from procedure B.

the agreement between the increase in activity of one specific enzyme protein and the *net* increase in amount of total protein was remarkably consistent. Increases in tryptophan synthetase activity of approximately 50% were associated with a rise in total protein content of 15 to 20%. The results obtained in experiments with the synthetic reagent solutions were inconsistent.

Discussion

The increases of apparent tryptophan synthetase activity observed in this study probably represent net syntheses of tryptophan synthetase enzyme protein.

With one notable exception, the properties of the material assayed as apparent tryptophan synthetase agree with those reported by Yanofsky (22). The enzyme appears in the same fractions during isolation procedures and is heat labile. The reaction it catalyzes requires the presence of L-serine, is inhibited in the presence of amino acids, and shows a specific requirement for pyridoxal phosphate. Further, our results (Table IV) suggest the presence in our extracts of the enzymically inactive tryptophan synthetase – inhibitor complex of Suskind and Kurek (28).

The notable difference between the properties of our crude preparations and those of highly purified tryptophan synthetase is in the much greater concentration of pyridoxal phosphate required by our preparations. This difference poses the question of whether the apparent tryptophan synthetase activity of our preparations is due to some species of protein molecule other than the enzyme described by Yanofsky (22), or whether the increased requirement for

cofactor is due to destruction of the coenzyme during the course of the assay. A definitive answer to the question should be forthcoming from current immunological studies.

Nevertheless, three lines of evidence indicate that we are dealing with the enzyme described by Yanofsky (22). Firstly, preparations precipitated from comparable extracts of growing mycelium of the 74A strain of *N. crassa* require the same high concentration of pyridoxal phosphate for maximal enzymic activity as do our conidial extracts (unpublished experiments). Secondly, we have prepared extracts from conidia of the td_2 mutant strain of *N. crassa*, in which the mutation is located in the compound locus controlling formation of tryptophan synthetase enzyme (25, 26, 27). These extracts neither contain nor develop any detectable tryptophan synthetase activity (31). Finally, in some of our experiments the apparent enzymic activity declined during the course of the assay. This finding is open to many possible interpretations, but is consistent with the suggestion that our crude enzyme preparations destroy essential pyridoxal phosphate during the assay of tryptophan synthetase activity.

The increases of activity occur under conditions favoring a net synthesis of total protein in the system (Table XI), and cannot readily be attributed to destruction of some inhibitor of the activity of pre-existing enzyme (Tables III and IV). In addition, 15 amino acids are required for the consistent development of maximal levels of tryptophan synthetase activity by our crude extracts (Table VIII), which makes it unlikely that we are dealing with a process of activation of some pre-existing complex precursor similar to that reported by Ullmann and Straub (8, 32) in the case of pancreatic amylase.

In addition, the requirements for formation of new enzymic activity, and the responses of the system to the addition of inhibitors are similar to those reported for other systems (1–19). Indeed, the system developed for *N. crassa* appears to be remarkably sensitive to the action of inhibitors. All of the inhibitors tested in this study, including nuclease enzymes, have also been found to inhibit either the synthesis of protein, or the incorporation of amino acids in some of the other reported systems. However, none of these latter systems has been reported to be sensitive to all the inhibitors.

The experience gained with the system described here has permitted us to develop an analogous system using extracts prepared from growing mycelium (unpublished results). However, it seems very likely that the conidial system will continue to be of importance in the analysis of many interesting genetic phenomena (31), and other problems related to the early growth stages of *N. crassa*.

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PREPARATION OF CAFFEIC AND DIHYDROCAFFEIC ACIDS BY METHODS SUITABLE FOR INTRODUCTION OF C^{14} INTO THE β-POSITION[1]

A. C. NEISH

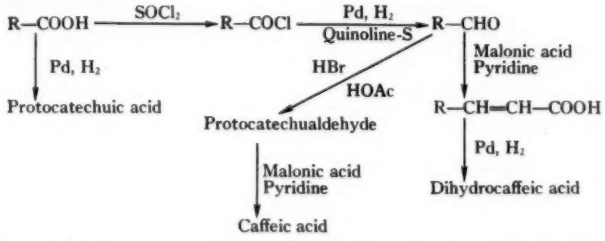
Abstract

3,4-(Dibenzyloxy)-benzoyl chloride (m.p. 95°) was prepared from the corresponding acid, and reduced to 3,4-(dibenzyloxy)-benzaldehyde (m.p. 86°) by Rosenmund's method. The over-all yield of this aldehyde was 52%, based on the barium carbonate used for preparation of the acid. The aldehyde was debenzylated by hydrogen bromide in acetic acid, and the protocatechualdehyde thus obtained was condensed with malonic acid, to give caffeic acid. Condensation of 3,4-(dibenzyloxy)-benzaldehyde with malonic acid gave 3,4-(dibenzyloxy)-cinnamic acid (m.p. 206–208°) which was converted to dihydrocaffeic acid by catalytic hydrogenation. The over-all yields of caffeic and dihydrocaffeic acids were 36% and 39%, respectively, based on barium carbonate. Protocatechuic acid was readily obtained by hydrogenolysis of 3,4-(dibenzyloxy)-benzoic acid; the yield was 70% based on carbonate.

Caffeic acid is of widespread occurrence in plants (1). It is found as the free acid and also as depsides such as chlorogenic acid and its isomers (2, 3) and as other alkali-labile compounds of unknown constitution (4). Tracer studies have shown that caffeic acid may be a member of a metabolic pool of phenylpropanoid compounds, from which intermediates are drawn for the biosynthesis of lignin (4, 5).

Caffeic acid-β-C^{14} has been prepared for studies on the biosynthesis of lignin (5). The route used was similar to that described below, but employed different blocking groups. The over-all yield, based on $BaC^{14}O_3$, was only 11%. This yield has been increased to 36%, using the method described in this communication. In addition, procedures for preparing dihydrocaffeic acid, protocatechuic acid, and protocatechualdehyde are given. These preparations are all based on 3,4-(dibenzyloxy)-benzoic acid which can be made, labelled in the carboxyl group, in 74% yields based on $BaC^{14}O_3$(6).

The syntheses described in this paper are as follows: where R = 3,4-(dibenzyloxy)-phenyl:



The over-all yields are shown in Table I; they are calculated from the barium carbonate used in preparation of R—COOH (6). The yields obtained

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TABLE I

Yields of products

Product	% yield, based on barium carbonate
3,4-(Dibenzyloxy)-benzoic acid*	74
3,4-(Dibenzyloxy)-benzoyl chloride	68
3,4-(Dibenzyloxy)-benzaldehyde	52
3,4-(Dibenzyloxy)-cinnamic acid	48
Protocatechuic acid	70
Protocatechualdehyde	48†
Caffeic acid	36
Dihydrocaffeic acid	39

*See reference 6.
†This yield refers to practical grade material, m.p. 146–152°. The yield of purified protocatechualdehyde was about 30%. The yields of all other compounds are based on the purified materials.

for each individual step were 76–93%. Each reaction was tested at least twice and concordant results were obtained.

This general route has been used for synthesis of β-labelled cinnamic acid (7) and a number of its derivatives, such as *p*-coumaric acid (5), ferulic acid (8, 23), glucoferulic acid (9), and sinapic acid (8, 24). The reduction of the acid chloride to the aldehyde is the step most likely to cause trouble; an apparatus which has been found to give reliable results is described below (Fig. 2). Generally speaking, the Rosenmund reduction (10) is the simplest method for converting an aromatic acid to its aldehyde. However, other methods have been developed which may be preferable for certain acids (11, 12).

In this laboratory the Rosenmund reduction has been found to give good results with benzoylchloride (7), *p*-acetoxybenzoyl chloride (5), and O-acetyl syringoyl chloride (5) using the procedure described below for 3,4-(dibenzyloxy)-benzoyl chloride. The reductions required only 1–2 hours and yields in excess of 75% were obtained with all the aryl–acyl chlorides tried. If the acid chloride is purified by distillation or recrystallization and if the apparatus is designed to prevent any material other than glass or Teflon from coming in contact with the reaction mixture, then the Rosenmund reduction should give reliable results.

The debenzylation of 3,4-(dibenzyloxy)-benzaldehyde required some study. Refluxing with aqueous 5 *N* hydrochloric or hydrobromic acids gave rather poor yields of protocatechualdehyde (less than 40%). Attempts were made to remove the benzyl groups selectively by hydrogenolysis without reducing the aldehyde group. These experiments were not encouraging, although protocatechualdehyde was obtained in 17% yield by hydrogenation in an alkaline medium. Protocatechualcohol was isolated (yield 42%) in one of these experiments. These experiments were discontinued when it was found that debenzylation proceeded smoothly at room temperature, using hydrogen bromide dissolved in glacial acetic acid, to give high yields of protocatechualdehyde.

Experimental

Reagents

The Pd (10%) – carbon catalyst was prepared using Darco-G-60 charcoal which had been purified by digestion with 10 parts of 5 *N* hydrochloric acid (for 15 minutes on a steam bath) and then washed thoroughly with water and dried at 110°. This was palladized following the directions given by Mozingo for catalyst D (13).

The Pd (5%) – $BaSO_4$ catalyst, and the quinoline-S, for the Rosenmund reduction were prepared according to the directions of Mosettig (10). Benzene and xylene were dried by refluxing with powdered lithium hydride (Maywood Chemical Works) for 1 hour and then distilling in the absence of moisture (14). Thionyl chloride was purified by distillation from quinoline, and then redistillation from boiled linseed oil (15). Pyridine and piperidine were refluxed with barium oxide and redistilled (15). Aniline was redistilled under reduced pressure (about 20 mm). The 30% solution of hydrogen bromide in glacial acetic acid is commercially available.

Protocatechuic Acid

Hydrogenolysis (16) of the benzyl groups of 3,4-(dibenzyloxy)-benzoic acid proceeded readily in ethanol using the Pd–charcoal catalyst. The apparatus shown in Fig. 1 was used. 3,4-(Dibenzyloxy)-benzoic acid (0.68 g; 2.0 mmoles)

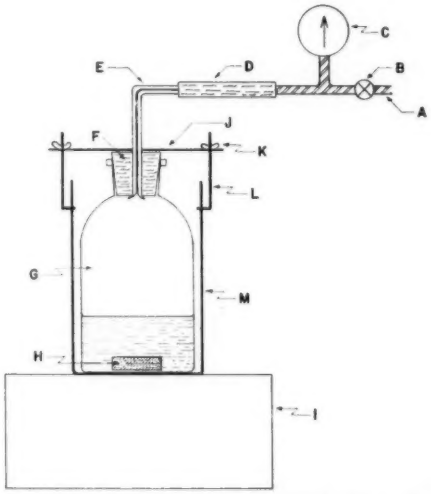

FIG. 1. Apparatus for catalytic hydrogenolysis of benzyl groups. The nipple A, stainless steel needle valve B, and pressure gauge C (range 0–60 lb/in²) are joined by standard ¼-in. stainless steel pipe fittings. D = rubber pressure tubing (¾-in. × 3/16-in.), E = 2-mm bore Pyrex tubing flared out below rubber stopper F, G = 125-ml Pyrex bottle, H = Teflon-coated magnetic stirrer bar, I = magnetic stirrer. The stopper is held in by brass bar J. J is notched to fit around E. K = wing nut, L = swiveled bolt fastened to brass case M.

from the lithium compound (6) was suspended in 25 ml of commercial absolute ethanol in the bottle G, and then 60 mg of catalyst was added. The apparatus

was assembled as shown and evacuated through A by a filter pump. It was then filled with hydrogen to a pressure of 26 lb/in^2, and B was closed. The mixture was stirred at room temperature and pressure readings were taken periodically. After 48 minutes the reaction was complete; the total drop in the pressure was 9.3 lb/in^2. The catalyst was filtered out, using Celite Analytical Filter Aid, and the filtrate was evaporated by a stream of air at room temperature. This gave 292 mg (95%) of cream-colored protocatechuic acid, m.p. 202–203°. A mixed melting point with an authentic sample (17) showed no depression.

The number of millimoles of hydrogen absorbed can be easily calculated from the pressure change provided the gas volume is known. It is difficult to measure this volume directly since the gauge contributes appreciably to it. The pressure drop noted above (9.3 lb/in^2) should correspond to absorption of 4.0 mmoles of hydrogen; therefore, for each millimole of hydrogen absorbed a pressure drop of 2.32 lb/in^2 should be observed, when 25 ml of liquid is used in G. This factor was used in calculating the moles of H_2 absorbed per mole of compound for the other hydrogenations.

3,4-(Dibenzyloxy)-benzoyl Chloride

A mixture of 1.00 g (2.99 mmoles) of 3,4-(dibenzyloxy)-benzoic acid (6) and 5 ml of thionyl chloride was heated for 90 minutes at 65° in a 250-ml Florence flask fitted with an air condenser capped by a drying tube. This is the temperature recommended by Billek and Herrmann (12) for preparation of 4-(benzyloxy)-benzoyl chloride. The solution was evaporated to dryness in a rotary evaporator (18) under reduced pressure, then 5 ml of dry benzene was added and evaporated, to aid in removal of the thionyl chloride. The crystalline residue was dissolved in about 30 ml of hot petrol (75–90°), the hot solution filtered through a sintered glass funnel, and the filtrate cooled at 3° for 2 hours. The solid was collected by suction, washed by 30–60° petrol, and dried *in vacuo* at 40° C. This gave 0.97 g (92%) of the acid chloride as very pale yellow-green needles, m.p. 95° C.

3,4-(Dibenzyloxy)-benzaldehyde

This compound was prepared by Rosenmund reduction (10) of the acid chloride, using the apparatus shown in Fig. 2. The hydrogen used was dried by a bubbler filled with concentrated sulphuric acid, and the exit gas was allowed to bubble through water to absorb the hydrochloric acid formed in the reduction. The reaction was followed by titration of the hydrochloric acid released with 0.200 *N* sodium hydroxide. 3,4-(Dibenzyloxy)-benzoyl chloride (0.97 g; 2.74 mmoles) was dissolved in 6 ml of dry xylene, containing 4 mg of quinoline-S (10), in the reaction vessel Q. The air was swept out by hydrogen while the solution was stirred, then 200 mg of Pd (5%) – $BaSO_4$ was added. The mixture was then stirred and refluxed vigorously while a continuous stream of hydrogen was passed through. The reduction required 2 hours, and 2.51 m.e. of hydrochloric acid (91.5%) was given off. The mixture was cooled with the hydrogen still flowing and then it was filtered through Celite Analytical Filter Aid contained in a sintered glass filter (porosity M). Ether

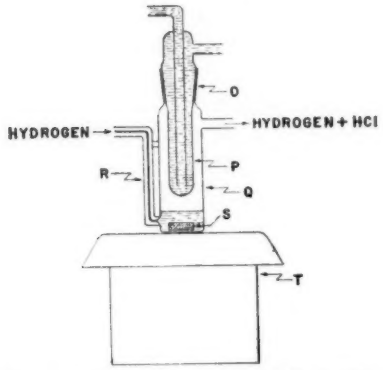

FIG. 2. Apparatus for Rosenmund reduction. O = 24/40 standard taper joint, P = cold finger condenser, Q = reaction vessel made from 30-mm O.D. Pyrex tubing, R = 1-mm bore Pyrex tubing, S = Teflon-coated magnetic stirrer bar, T = combination hot plate and magnetic stirrer.

was used to rinse out vessel Q and to wash the filter cake. The combined filtrate and washings (about 20 ml) was washed in a separatory funnel with 5 ml of 0.2 *N* sodium hydroxide and then with 5 ml of water. The ether layer was then evaporated by a stream of air at room temperature. This gave 0.82 g (94%) of crude 3,4-(dibenzyloxy)-benzaldehyde, mp. 80–85°. Recrystallization from ethanol gave 0.66 g (76%), m.p. 86°. An authentic sample, prepared by benzylation of protocatechualdehyde (19, 20) and recrystallized to constant melting point from ethanol, was also found to melt at 86° C. A mixed melting point showed no depression. Mahal *et al.* (20) found a melting point of 93° for this compound.

3,4-(Dibenzyloxy)-cinnamic Acid

A mixture of 1.28 g (4.0 mmoles) of 3,4-(dibenzyloxy)-benzaldehyde, 0.82 g (8.0 mmoles) of malonic acid, 4 ml of pyridine, and 0.4 ml of piperidine was refluxed for 4 hours using a reflux condenser protected by a drying tube. The mixture was cooled to room temperature and treated with 20 ml of water containing 6 ml of concentrated hydrochloric acid. After the mixture was stirred a few minutes at room temperature it was filtered and the solid washed with water. It was dissolved in about 40 ml of 93% acetic acid, the mixture cooled at 3° overnight, then filtered and the solid washed by 90% acetic acid and dried at 50° *in vacuo*. This gave 1.29 g (92%) of 3,4-(dibenzyloxy)-cinnamic acid, m.p. 206–208°. Recrystallization from 95% acetic acid did not raise the melting point. Microanalysis gave C = 76.83%, H = 5.65%. Calculated for $C_{23}H_{20}O_4$: C = 76.65, H = 5.59.

Dihydrocaffeic Acid

3,4-(Dibenzyloxy)-cinnamic acid (1.00 g; 2.86 mmoles) was hydrogenated in 25 ml of ethanol as described above for protocatechuic acid, except the amount of catalyst was increased to 150 mg. The reaction required 2 hours, the pressure drop was 19.9 lb/in^2. This corresponds to absorption of the theoretical

amount of hydrogen, i.e. 3.0 moles of hydrogen per mole of acid. The catalyst was filtered out and the filtrate evaporated by an air stream at room temperature. This gave 0.50 g (96%) of cream-colored dihydrocaffeic acid, m.p. 135–137°. Recrystallization from water gave 297 mg of colorless crystals, m.p. 139°. The mother liquors were evaporated and the residue was recrystallized from 4 ml of 14% acetic acid in benzene to yield another 129 mg, m.p. 139°. The total yield of purified dihydrocaffeic acid, m.p. 139°, was 423 mg (81%). A mixed melting point with an authentic sample, prepared by reduction of caffeic acid, showed no depression.

Protocatechualdehyde

3,4-(Dibenzyloxy)-benzaldehyde (0.64 g) was mixed with 5 ml of glacial acetic acid in a 60-ml glass-stoppered conical flask, then 5 ml of 30% hydrogen bromide in glacial acetic acid was added and the mixture was allowed to stand for 22 hours at room temperature (about 21°). The dark-red product was evaporated under reduced pressure, using a rotary evaporator (18), to remove the acetic acid and benzyl bromide. The solid residue was dissolved in ether (30–40 ml). This ether extract was washed with 3 to 4 ml of water in a separatory funnel and the washings discarded. The ether layer was then extracted by two 12-ml portions of 20% sodium bisulphite. The total bisulphite extract was treated with 10 ml of 10 *N* sulphuric acid and then aerated under reduced pressure until the sulphur dioxide was removed (about 45 minutes). The acidic solution was then extracted by three 30-ml portions of ether. The combined ether extracts were washed by 3–4 ml of 0.5 *M* sodium bicarbonate and followed by 3–4 ml of water. The ether was then evaporated by an air stream at room temperature and the residue dried at 50° *in vacuo*. This gave 258 mg (93%) of protocatechualdehyde as a light-brown crystalline solid. This material (m.p. 146–152°) was pure enough for the preparation of caffeic acid (see below). Protocatechualdehyde may be purified by recrystallization from water (19). However, the solubility in water is quite high and only about 60% was recovered, with m.p. 152–153°, when this was done. The melting point was not depressed when this material was mixed with an authentic sample (19).

Caffeic Acid

The protocatechualdehyde sample melting at 146–152° was used; 294 mg (1.80 mmoles) was mixed in a test tube with 0.35 g of malonic acid, 1.0 ml of dry pyridine, and 0.05 ml of redistilled aniline (5, 21). The mixture was protected by a drying tube. It was allowed to stand at room temperature for 20 hours, then kept at 50° for 5 hours, cooled to room temperature, and 9 ml of 2.5 *N* hydrochloric acid was added. The mixture was stirred to induce crystallization. It was kept at 0° for 1 hour, filtered, and the solid washed by cold water and dried at 60° *in vacuo*. This gave 259 mg of an orange solid which melted with decomposition at 206–211°. Another 13 mg, m.p. 206–209°, was obtained from the mother liquors. These crops were combined, dissolved in 4 ml of 0.5 *M* sodium bicarbonate, treated with a little charcoal, and filtered using 2 ml of wash water. The filtrate was acidified by 0.6 ml of 5 *N* hydrochloric acid and cooled at 3° for 3 hours. The crystalline solid was collected

by suction, washed with ice water, and dried as above. This gave 246 mg (76%) of cream-colored caffeic acid, m.p. 206–211°.

Hydrogenolysis of 3,4-(Dibenzyloxy)-benzaldehyde

Several attempts were made to remove the benzyl groups without reducing the aldehyde group. When 2 mmoles of 3,4-(dibenzyloxy)-benzaldehyde was treated as described above under "Protocatechuic acid", the reaction stopped after absorption of 3.7 mmoles of hydrogen per mmole of aldehyde. This amount of hydrogen is more than enough to remove both benzyl groups and reduce the aldehyde to the alcohol. Presumably some reduction of the alcohol to the hydrocarbon had occurred (16). The reaction mixture was worked up and 108 mg of a solid, m.p. 137–138°, was isolated. This melting point agrees with that recorded for protocatechualcohol by Rosenmund and Boehm (22).

This experiment was repeated using 20 ml of ethanol mixed with 5 ml of *N* hydrochloric acid as the solvent, and 100 mg of catalyst. A rapid absorption of hydrogen occurred at first and stopped after 100 minutes, when it corresponded to 3.94 mmoles of hydrogen per millimole of (dibenzyloxy)-benzaldehyde. This amount of hydrogen is enough for practically complete reduction to homocatechol, but the sirupy product did not crystallize.

A third experiment was run using *N* sodium hydroxide in place of hydrochloric acid. Absorption of hydrogen was slower than in the other experiments. The reaction was stopped when it corresponded to 1.82 mmoles per millimole of aldehyde. The reaction mixture was worked up by bisulphite extraction and 47 mg (17%) of crude protocatechualdehyde was obtained. This was not investigated further since the debenzylation with hydrogen bromide in acetic acid was found to give high yields of protocatechualdehyde.

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PREPARATION OF C^{14}-LABELLED ACIDS FROM $C^{14}O_2$ AND ARYL LITHIUM COMPOUNDS[1]

A. C. NEISH

Abstract

A simplified technique was developed for carbonation of Grignard reagents or organolithium compounds, with $C^{14}O_2$ generated from barium carbonate. 3,4-(Dibenzyloxy)-bromobenzene was treated with *n*-butyl lithium in ether and then with $C^{14}O_2$ to give 3,4-(dibenzyloxy)-benzoic acid-carboxyl-C^{14}. The yield was 74% based on barium carbonate. 2,6-Dichlorobenzoic acid-carboxyl-C^{14} (yield 77%) and 3,5-dichlorobenzoic acid-carboxyl-C^{14} (yield 76%) were prepared from the corresponding dichlorobromobenzenes by a similar sequence of reactions. Attempts to synthesize 2,4- and 2,5-dichlorobenzoic acids by this route gave poor yields of unidentified acids, from which the expected products could not be isolated.

The synthesis of carboxylic acids by reaction of carbon dioxide with Grignard reagents has been used frequently as a step in the preparation of C^{14}-labelled compounds (1). The method is important, particularly since C^{14} is least expensive when purchased as a carbonate. Unfortunately some substituted aryl bromides fail to react with magnesium so it is necessary to form the lithium compounds instead. This is best done by an exchange reaction with *n*-butyl lithium in ether (2). The reaction sequence then becomes:

$$R\text{—}Br \xrightarrow[\text{lithium}]{n\text{-butyl}} R\text{—}Li \xrightarrow[\text{dioxide}]{\text{carbon}} R\text{—}COOH$$

where R is a substituted phenyl radical.

It is preferable to use the Grignard reagent when possible because of the highly reactive nature of organolithium compounds (2, 3). However, a number of acids have been prepared in good yields from aryl lithium compounds (2). Some examples where $C^{14}O_2$ was used include veratric acid (1), 3-methoxy-4-benzyloxybenzoic acid (4), 4-(benzyloxy)-benzoic acid (5), 3,5-dimethoxy-4-(benzyloxy)-benzoic acid (6), and 3,4,5-trimethoxybenzoic acid (7).

The present communication describes the preparation of 3,4-(dibenzyloxy)-benzoic acid from the corresponding bromo compound via the lithium compound; the yield was 74% based on $C^{14}O_2$. This acid should be useful as an intermediate in the synthesis of C^{14}-labelled 3,4-dihydroxyphenyl compounds, and its application for some preparations is described elsewhere (8).

The exchange reaction between *n*-butyl lithium and aryl halides is rapid with bromo- or iodo- compounds but quite slow with the chloro- compounds (2). Thus Gilman and Spatz (9) obtained good yields of 3-chlorobenzoic acid by carbonation of the aryl lithium obtained from *m*-chlorobromobenzene. In the present investigation it was found that 2,6- and 3,5-dichlorobenzoic acids could be obtained from the corresponding bromo compounds, by this route, in good yields (76–77%) based on $BaC^{14}O_3$. The reaction failed, how-

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ever, when applied to the preparation of the 2,4- and 2,5-dichlorobenzoic acids. The synthesis of the 2,3- and 3,4-isomers was not investigated. The dichlorobenzoic acids are of biochemical interest because certain isomers act as plant growth substances, while others do not (10).

The preparations of the dichloro- and the dibenzyloxy-benzoic acids are described in detail in this communication; as far as the author is aware these compounds have not been prepared before by a route as suitable for C^{14} labelling. It was necessary to use the organolithium compounds since the aryl bromides would not form the Grignard reagents. The apparatus and techniques used are simpler than those which are described in detail by other workers for reaction of $C^{14}O_2$ with metal organic compounds (1, 4). The conditions used for the preparation of 3,4-(dibenzyloxy)-benzoic acid are essentially the same as given by Kratzl and Billek (4) for 3-methoxy-4-(benzyloxy)-benzoic acid-carboxyl-C^{14}. The preparation of the latter compound was repeated using the techniques described below. The yield of recrystallized acid (m.p. 172–173°) was 72%, or practically the same as reported by Kratzl and Billek.

Acetic acid-1-C^{14} was prepared by carbonation of methyl magnesium iodide (11), using the method of generating $C^{14}O_2$ described in this paper. The yield, based on barium carbonate, was 95%; so there was little or no loss in generating and transferring the carbon dioxide. Carboxyl-labelled benzoic acid (yield 80%) and *p*-anisic acid (yield 85%) were also prepared (11) from phenyl magnesium bromide and *p*-methoxyphenyl magnesium bromide, respectively. These yields compare favorably to those obtained by a more complicated *modus operandi* (1).

Experimental

Analytical Methods

Melting points of compounds melting below 100° were determined by the classical capillary tube technique. The melting points of the other compounds were measured by the Fisher–Johns hot-stage apparatus (Fisher Scientific Company). All melting points were corrected.

The Model 6000 Dynacon Electrometer (Nuclear-Chicago Corporation) was used for determination of C^{14}. The samples were converted to carbon dioxide and measured in a 250-ml ion chamber.

Apparatus

The apparatus used for preparation and carbonation of organolithium compounds is illustrated in Fig. 1. The vacuum was supplied by an oil pump protected by a vapor trap cooled by dry ice. A Macleod gauge was connected to a side-arm between the pump and the cold trap. A diffusion pump was not used since a pressure of 0.01 mm of Hg is satisfactory for transferring carbon dioxide. The use of the vacuum line technique for handling radioactive compounds has been described in detail by Calvin *et al.* (1). Operation of the apparatus (Fig. 1) is described below under "2,6-Dichlorobenzoic acid-carboxyl-C^{14}". All joints were lubricated with high vacuum grease, and were held together by springs which are not shown in the drawing.

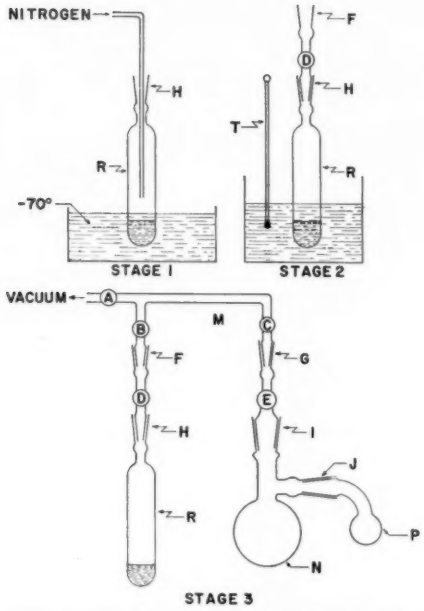

FIG. 1. Preparation and carbonation of aryl lithium compounds.
Stage 1. Reaction tube being flushed just before addition of *n*-butyl lithium.
Stage 2. Exchange reaction between aryl bromide and butyl lithium.
Stage 3. Generation and transfer of $C^{14}O_2$. The tube R is cooled by liquid nitrogen.
A = 10-mm bore stopcock; B, C, D, and E = 3-mm bore stopcocks; F, G, H, and J = 14/35 standard taper joints; I = 19/38 standard taper joint; M = Pyrex manifold tube (18-mm diameter); N = 500-ml flask; P = 50-ml flask; R = 250-ml reaction tube; T = low-temperature thermometer.

Reagents

Sodium carbonate-C^{14} was purchased as a solution in dilute sodium hydroxide from Atomic Energy of Canada Limited. Unlabelled sodium carbonate was added (1 mmoles per 2 mc) and then the carbonate was precipitated by addition of barium chloride. The precipitate ($BaC^{14}O_3$) was washed successively with water, ethanol, and ether, air-dried on the filter, then dried for several hours at 110° C and stored in a desiccator.

Dry, oxygen-free nitrogen was obtained by passing commercial nitrogen through Fieser's solution (12) and then through two towers packed with anhydrous calcium chloride. Ether (reagent grade) was purified by refluxing 1 hour with powdered lithium hydride (Maywood Chemical Works) and then distilling in the absence of moisture (13). A solution of *n*-butyl lithium in purified ether was prepared as described elsewhere (2, 3). It was filtered through a bed of dry Celite Analytical Filter Aid contained in a sintered glass funnel (porosity M). The filtrate was caught in a 250-ml round-bottom two-neck flask having glass joints. One neck was fitted with an adapter having a stopcock and the funnel was held by a rubber stopper inserted in the other neck. Suction was applied intermittently through the stopcock from a filter

pump. A steady suction caused excessive boiling of the filtrate. When the filtration was finished the flask was filled with purified nitrogen through the stopcock and the funnel was replaced by a glass stopper lubricated with silicone grease.

This solution of *n*-butyl lithium was stored at 3° and analyzed (2, 3) each time it was used. It may be kept for several weeks. Samples were removed by a pipette equipped with a rubber bulb; purified nitrogen was passed over the surface of the solution while this operation was carried out.

Dichlorobromobenzenes

A procedure (14) for the preparation of 2-chlorobromobenzene from 2-chloroaniline was used for preparation of 2,6-dichlorobromobenzene from 2,6-dichloroaniline (15). It involved diazotization followed by treatment with cuprous bromide and steam distillation. The crystalline product was filtered from the distillate and purified by recrystallization from ethanol. The yield of 2,6-dichlorobromobenzene (m.p. 65°) was about 48%, based on the dichloroaniline.

The 2,4- and 2,5-dichloroanilines (Eastman Kodak Co.) were converted to the corresponding dichlorobromobenzenes in the same way. The products were extracted from the steam distillates by ether and purified by fractional distillation at 30-mm pressure. 2,4-Dichlorobromobenzene (m.p. 24°) was obtained in a yield of 40%, and 2,5-dichlorobromobenzene (m.p. 34°) in a yield of 45%, based on the technical grade dichloroanilines.

3,5-Dichlorobromobenzene (16) was prepared from 2,4-dichloroaniline by bromination followed by replacement of the amino group with hydrogen. 2,4-Dichloroaniline (35.6 g) was treated as described for preparation of symmetrical tribromobenzene from aniline (17) using 1.1 molecular proportions of bromine. The crude 2,4-dichloro-6-bromoaniline was filtered out, washed by water, and the moist filter cake dissolved in 425 ml of 95% ethanol mixed with 105 ml of benzene. The mixture was warmed to effect solution and concentrated sulphuric acid (28 ml) was added; then sodium nitrite (28 g) was added as fast as the violence of the reaction would allow (17). The mixture was refluxed 1 hour and allowed to stand at room temperature overnight; then 250 ml of solvent was distilled out and the residue treated with 250 ml of water and extracted by ether. The extract was evaporated and the residue steam-distilled. A yellow solid (40.5 g; m.p. 50–60°) was filtered from the distillate. Three recrystallizations from ethanol gave 15.0 g of 3,5-dichlorobromobenzene, m.p. 75° C. The yield was 30% based on the dichloroaniline.

2,6-Dichlorobenzoic Acid-carboxyl-C^{14}

This preparation may be used to illustrate the general method (see Fig. 1). A solution of 1.02 g (4.5 mmoles) of 2,6-dichlorobromobenzene in 30 ml of purified ether was prepared in the reaction tube. The tube was flushed out with dry, oxygen-free nitrogen and cooled to about −70° in a dry ice – methanol bath (Fig. 1, stage 1). A solution of *n*-butyl lithium in ether (2 ml; 1.65 *N*) was then pipetted in, and the tube closed by an adapter and transferred to a methanol bath, as shown in Fig. 1 (stage 2). The bath temperature was

maintained at −35° to −45° by addition of small pieces of dry ice, as required. The exchange reaction was allowed to proceed for 15 minutes at this temperature with occasional shaking, by hand. The reaction tube was then attached to the vacuum manifold, as shown in Fig. 1 (stage 3) and cooled at once by liquid nitrogen. After the contents were frozen, B and D were opened and the system was evacuated to about 10-micron pressure.

The $C^{14}O_2$ may be generated either before or after the exchange reaction, whichever is most convenient. Dry $BaC^{14}O_3$ (600 mg; 3.04 mmoles; 2940 μc) was put in flask N, and flask P was charged with 25 ml of concentrated sulphuric acid (reagent grade). The apparatus was assembled as shown (Fig. 1, stage 3) and the system evacuated with all stopcocks open. When the pressure reached 10 microns, C and E were closed and then P was revolved one-half turn around J, in order to mix the sulphuric acid with the barium carbonate. The carbon dioxide generator was then detached at G, with C and E still closed. A greased 14/35 standard taper joint is easily pulled apart, even under full vacuum. The generator assembly was gently shaken and rotated, by hand, to make sure all of the carbonate was wetted. The pressure inside the flask was below atmospheric pressure so there was no danger of $C^{14}O_2$ escaping.

The apparatus was reassembled as shown in Fig. 1 (stage 3) and evacuated to 10 microns with C open but E still closed. Stopcock A was then closed and E was opened to permit the $C^{14}O_2$ to diffuse into the reaction tube; about 15 minutes was allowed for diffusion. Flask N was warmed by a flame during the latter stages, to drive the carbon dioxide out of solution. When diffusion was completed D and B were closed and the reaction tube detached at F and transferred to a dry ice – methanol bath containing excess dry ice (−70°). The carbonation was allowed to proceed for 15 minutes, the tube being shaken by hand for a few seconds at 1- to 2-minute intervals.

After the carbonation was finished 5 *N* hydrochloric acid (10 ml) was added through D and the contents of the reaction tube shaken and then transferred to a separatory funnel. Water and ether were used to rinse out the tube. The aqueous phase was discarded and the ether layer washed once with water and then extracted twice with 20-ml portions of 0.5 *N* NaOH. The alkaline extract was acidified with 5 *N* hydrochloric acid (7 ml) and the colorless oil that separated was extracted back into ether. This ether extract was dried by anhydrous sodium sulphate and evaporated at room temperature. The oily residue was taken up in a few milliliters of ethanol and this solution was evaporated by an air stream at room temperature. The residue was treated with 1 to 2 ml of hexane; slow evaporation at room temperature gave a colorless solid, 0.51 g, m.p. 139–141°. This was sublimed, under the full vacuum of an oil pump, at a bath temperature of 100–110°. The 2,6-dichlorobenzoic acid-carboxyl-C^{14} was obtained as a colorless sublimate, m.p. 142–144°. The yield was 0.46 g, or 77% based on $BaC^{14}O_3$. A mixed melting point with an authentic sample, m.p. 142–145° (10) showed no depression. The same yield was obtained in a run with unlabelled barium carbonate.

3,5-Dichlorobenzoic Acid-carboxyl-C^{14}

The lithium compound was prepared from 0.85 g (3.74 mmoles of 3,5-dichlorobromobenzene in 30 ml of ether, and *n*-butyl lithium (3.1 mmoles) by the technique described for 2,6-dichlorobenzoic acid. The exchange reaction was run for 12 minutes at −35°. Carbonation was effected, as described above, using $C^{14}O_2$ from 666 mg (3.38 mmoles; 2980 μc) of $BaC^{13}O_3$. On acidification of the alkaline extract a colorless, crystalline solid precipitated. After the mixture was cooled at 0° for 30 minutes it was filtered and the solid washed with water and dried at 60° *in vacuo* for 45 minutes. This gave 0.49 g (76%) of a colorless solid (m.p. 188–189°), which was recrystallized from dilute ethanol to give 0.44 g with the melting point unchanged. A mixed melting point determination, with an authentic sample (10) having the same melting point, showed no depression. The specific activity was 4610 μc per g. A sample of carrier (0.69 g) was dissolved in the mother liquors and crystallized out giving 0.62 g, m.p. 188–189°, with a specific activity of 368 μc/g. The total C^{14} recovered as 3,5-dichlorobenzoic acid was thus 2260 μc (75.6%).

Attempted Preparation of 2,4- and 2,5-Dichlorobenzoic Acids

2,4-Dichlorobenzene (4.5 mmoles) was treated with 3.5 mmoles of *n*-butyl lithium as described above for the 2,6-isomer. Carbonation gave only 0.16 g of a sirupy acid fraction. Vacuum sublimation at 110° gave a few milligrams of an impure sublimate. When the synthesis was repeated, and the exchange reaction run at room temperature for 3 to 4 minutes, no acids were formed by carbonation.

2,5-Dichlorobromobenzene (5.3 mmoles) was also treated with *n*-butyl lithium (4.4 mmoles) and carbonated with 4.3 mmoles of CO^2, as described above for the 2,6-isomer. The acid fraction was 0.32 g of a solid melting over a wide range (152–177°). It was only slightly soluble in boiling water. Fractional crystallization from dilute ethanol or from hexane–benzene mixtures gave solids melting over a range of 30° or more. Vacuum sublimation at 115° gave 73 mg of sublimate which, on crystallization from petrol, had a melting range of 153–167°. Apparently a mixture of acids was obtained which contains little, if any, 2,5-dichlorobenzoic acid.

3,4-Dibenzyloxybromobenzene

Salicylaldehyde (50 g) in 100 ml of glacial acetic acid was brominated as described by Auwers and Bürger (18). The product was recrystallized from 98% ethanol to give 34 g (41%) of 5-bromosalicylaldehyde, m.p. 105°.

This aldehyde (29.8 g) was triturated with 149 ml of *N* sodium hydroxide at room temperature until it was nearly all dissolved, then 224 ml of 1.66 *N* hydrogen peroxide (temp. 3°) was added all at once (19). The mixture was allowed to stand 1 hour at room temperature, then neutralized with a little sulphuric acid, and the product extracted by ether (three portions). The combined ether extract was washed with 20% sodium bisulphite, the ether was evaporated, and the residue dried at room temperature and recrystallized from carbon tetrachloride. This gave 24.7 g (88%) of 3,4-*dihydroxybromobenzene* (19), m.p. 84°. Benzylation of 3,4-dihydroxybromobenzene (12.4 g) was accomplished

by refluxing it for 14 hours with benzyl chloride (16 ml), anhydrous potassium carbonate (42 g), and 200 ml of anhydrous acetone. Water (250 ml) was then added and the mixture distilled until the head temperature reached 90°, in order to remove the acetone. The residue was cooled, extracted by ether, and the extract washed with three 50-ml portions of *N* sodium hydroxide, then with water, dried by anhydrous sodium sulphate, evaporated at room temperature, and the residue crystallized from commercial absolute ethanol. This gave 10.6 g (44%) of 3,4-(dibenzyloxy)-bromobenzene, m.p. 64–65°. It was recrystallized from ethanol to give 9.7 g, m.p. 66°. Microanalysis gave C = 65.11%, H = 4.82%, and Br = 21.46%. Calculated for $C_{20}H_{17}O_2Br$: C = 65.05%, H = 4.64%, and Br = 21.64%.

3,4-(Dibenzyloxy)-benzoic Acid-carboxyl-C^{14} from 3,4-(Dibenzyloxy)-bromobenzene

This acid was prepared by the same general methods described above for preparation of 2,6-dichlorobenzoic acid. A solution of 2.58 g (7 mmoles) of 3,4-(dibenzyloxy)-bromobenzene in 40 ml of purified ether (Fig. 1, stage 1) was treated with 4.95 mmoles of *n*-butyl lithium. The exchange reaction was run for 15 minutes in an ice–ethanol bath at −10° to −15° (Fig. 1, stage 2), and carbonation was effected by $C^{14}O_2$ generated from 890 mg of $BaC^{14}O_3$ (4.50 mmoles; 5100 μc).

Acidification, after carbonation was completed, gave an insoluble solid floating in the ether layer. The clear aqueous layer was drawn off and discarded. The ether layer was washed once with water and then extracted with two 20-ml portions of *N* sodium hydroxide. The clear alkaline extract was acidified by concentrated hydrochloric acid (6 ml), cooled at 0° for 30 minutes, and the colorless solid was filtered out, washed by water, and dried at 60° *in vacuo*. This crude acid (1.26 g, m.p. 170–184°) was recrystallized from ethanol to give 1.02 g of 3,4-(dibenzyloxy)-benzoic acid-carboxyl-C^{14}, m.p. 186°, with a specific activity of 3460 μc per g. A sample of the unlabelled acid (2.0 g) was recrystallized from the alcoholic mother liquors. This gave 1.74 g, m.p. 186°, containing 154 μc per g. The total yield was thus 3790 μc or 74.4% based on $BaC^{14}O_3$.

The melting point was not raised by further recrystallizations. An unlabelled sample prepared in the same way gave the same yield. Analysis of this sample gave: C = 75.52%, H = 5.43%. Calculated for $C_{21}H_{18}O_4$: C = 75.43, H = 5.53. A mixed melting point with the sample prepared by benzylation of protocatechuic acid (see below) showed no depression.

The aqueous mother liquors from the crude 3,4-dibenzyloxybenzoic acid had a pronounced odor of valeric acid. Steam distillation gave a volatile acid fraction containing 202 μc, or 4% of the C^{14} used. This is a measure of the amount of *n*-butyl lithium present at the end of the exchange reaction. There was no detectable odor of valeric acid in synthesis of the dichlorobenzoic acids.

3,4-(Dibenzyloxy)-benzoic Acid from Protocatechuic Acid

A sample of this compound was prepared to check the identity of the product obtained by carbonation of the aryl lithium compound (see above). Priestley

and Moness (20) obtained a small amount of 3,4-(dibenzyloxy)-benzoic acid by degradation of 3,4-(dibenzyloxy)-propiophenone. However, benzylation of protocatechuic acid is preferable as a preparative method.

A mixture of 15.4 g of protocatechuic acid (21), 165 ml of ethanol, 100 ml of water, 12.1 g of sodium hydroxide, and 38 ml of benzyl chloride was refluxed for 3 hours and then kept at 3° overnight. The mixture was adjusted to pH 8 and extracted by ether. The extract was discarded and the aqueous residue was acidified by hydrochloric acid (pH about 1.5). The precipitate was filtered out, washed by water, and air-dried. The crude product (8.6 g; m.p. 130–150°) was purified by three recrystallizations from ethanol. The purified product (4.4 g; 13%) was composed of long colorless needles, m.p. 186°. Priestley and Moness (20) reported m.p. 182°.

Acknowledgments

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LOSS OF PHOSPHOLIPID IN THE SKIN OF TUMOR-BEARING RATS[1]

ELDON M. BOYD AND IRENE E. MILLAR

Abstract

There occurs a unique, statistically significant fall in the level of phospholipid in the skin of albino rats bearing large Walker carcinosarcomata 256. The shift is similar to that produced by application of methylcholanthrene. It is preceded by loss of skin weight and increases in levels of skin water, cholesterol, and cholestrol esters. It is accompanied by loss of skin neutral fat and cholesterol esters. The quadratic shift in cholesterol esters occurs in hair *before* it occurs in skin, and is accompanied by a loss of hair weight and a slight but significant rise in levels of hair neutral fat. Declining levels of skin phospholipid may be part of the mechanism of production of the sallow appearance of the skin in late malignancy.

As Walker carcinosarcoma 256 grows in the body of rats, there occurs a significant loss of weight in most organs of the host. Along with loss of weight, there occur significant increases in the levels of water, phospholipid, and cholesterol, and decreases in the level of neutral fat (1). This series of changes is particularly marked in fat-storing organs, such as mesentery (2), and might be expected to be marked in skin. A fall in the level of neutral fat has been reported in skin but shifts in the levels of cholesterol, phospholipid, and water were not found to be statistically significant (3). A fall in the level of polyunsaturated fatty acids, which normally comprise some 2% of skin lipids, has been reported in skin of rats bearing the Walker tumor (4).

It appeared possible that failure to demonstrate significant shifts in the levels of phospholipid, cholesterol, and water in the skin of tumor-bearing rats might have been due to failure to include studies upon rats bearing tumors of a sufficiently wide range of weight. It has been demonstrated, in the thymus gland for example (5), that shifts in levels of phospholipid and cholesterol can occur over, but not below or above, a certain range of tumor weight. Accordingly, weight, water, and lipids were measured upon the skin of albino rats bearing tumors which extended in weight from 1 to 150% of host (minus tumor) weight.

Hair is continuously produced in the rat from the follicular bulb, which, as part of the germinal epithelium, has the ability to accumulate lipids (6). Accumulation of lipid is marked during the anagen or growth period of the hair follicle (6). It appeared possible, therefore, that a change in the lipid content of skin in animals bearing small tumors might be reflected a week or two later in hair.

To compare quantitative differences in skin (and hair) with what had taken place in the rest of the body, residual carcass was analyzed. Residual carcass in this project was the whole body minus the gastrointestinal tract, skin, hair, and, in the tumor bearers, the tumor.

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Contribution from the Department of Pharmacology, Queen's University, Kingston, Ontario, Canada. This project was assisted by grants from the National Cancer Institute of Canada and from Parke, Davis and Company Limited.

Method

The experiments were performed upon 106 albino rats of a Wistar strain which has been bred since 1937 in the animal quarters of the Department of Pharmacology at Queen's University. The animals were used shortly after weaning, at 5 to 7 weeks of age. They were divided into 53 littermate pairs, including 28 pairs of twin males and 25 pairs of twin females. One of each pair of twins was inoculated aseptically in the inguinal region with 20 to 30 mg of fresh, nonnecrotic Walker carcinosarcoma 256. The animals were fed Purina fox chow checkers and water ad libitum.

The pairs of animals were killed at intervals after 1 to 6 weeks of tumor growth. They were weighed before and after removal of the tumor. The hair was removed by Wahl clippers and the skin peeled off the entire animal. The gastrointestinal tract was removed and the residual carcass homogenized in a Waring blendor. Water and lipid levels were measured by methods previously described (see, for example (3)) and expressed as grams per 100 g nonlipid dry weight of tissue. The data were plotted and subjected to "t" tests of the significance of differences between nonindependent samples and to analysis of regression against tumor weight (7). Tumor weight was expressed as percentage of host (minus tumor) weight.

Results

Measurements upon the control twins are summarized in Table I and upon the tumor bearers in Table II.

TABLE I

Measurements upon non-tumor-bearing twins*

Measurement	Skin	Hair	Residual carcass
Gram fresh weight	29.1 ±10.3	2.24 ±0.753	130 ±35.0
Water	222 ±37.2	10.1 ±1.90	268 ±26.2
Neutral fat	19.4 ±13.6	1.39 ±0.464	12.1 ± 4.92
Phospholipid	2.02 ± 0.683	0.146±0.112	3.92 ± 1.19
Free cholesterol	0.516± 0.289	0.295±0.059	0.527± 0.152
Cholesterol esters	0.125± 0.402	0.214±0.146	0.019± 0.080

*Levels of water and lipids were measured as grams per 100 g nonlipid dry weight of tissue. The results are expressed as mean ± standard deviation.

TABLE II

Significant ($P < 0.05$) differences in tumor-bearing rats*

	Skin			Hair			Residual carcass		
Measurement	%	Range of tumor weight	N	%	Range of tumor weight	N	%	Range of tumor weight	N
Gram fresh weight	− 39	16 to 150	43	−9	1 to 150	49	−18	17 to 150	42
Water	+ 14	22 to 59	19	None			+14	19 to 150	37
Neutral fat	− 62	48 to 150	20	+16	16 to 59	21	−54	19 to 150	37
Phospholipid	− 21	55 to 150	18	None			+19	48 to 150	20
Free cholesterol	+ 40	41 to 59	10	None			+19	22 to 87	30
Cholesterol esters	+135	22 to 71	23	+41	1 to 22	16	None		
	− 99	71 to 150	10	−34	34 to 134	23			

*Differences are expressed as $(\bar{X}d/\bar{X}c)\times 100$, where $\bar{X}d$ is the mean of $Xt - Xc$, Xt the value in a tumor-bearing rat, and Xc the corresponding value in its control twin. Tumor weight is expressed as a percentage of the weight of host minus its tumor. Differences were not significant outside the range of tumor weight noted above.

There was loss of weight of skin, hair, and residual carcass throughout most of tumor growth. Loss of weight in skin was greater than in hair ($P<0.001$) or residual carcass ($P=0.02$), and was negatively correlated with log tumor weight, the estimated loss in grams being (4.39–9.28 log tumor weight) $\pm$ 8.07.

Water levels of skin were increased only in animals bearing tumors within the range of 22 to 59% of host weight. There were 19 animals in this group. Regression of $Xt-Xc$ of all animals on tumor weight was secondary parabolic with a negative value for c in the estimating equation (8), Xt being the value in the tumor bearer and Xc the corresponding value in its control twin. There was no significant change in the water level of hair. In residual carcass, water levels increased at tumor weights of 10 to 30% of host weight and remained elevated with little further increase as the tumor grew.

The level of neutral fat fell to about the same extent in skin as in residual carcass, the loss occurring somewhat later in tumor growth in the case of skin. Loss of neutral fat in skin was negatively correlated with log tumor weight. There was a small but significant rise in levels of neutral fat in hair (see Table II) at tumor weights of about 15 to 60% of host weight, and the regression line was a secondary parabola.

Levels of phospholipid declined in skin, rose in residual carcass, and remained unchanged in hair. Scatter plots revealed that the decline in skin phospholipid levels appeared at tumor weights equal to some 40 to 60% of host weight. Regression in skin was negatively correlated with log tumor weight.

As may be seen by data given in Table II, the regression of levels of free cholesterol on tumor weight was similar to that of water.

Skin and hair normally contain rather large amounts of cholesterol esters (Table I). In both organs, levels of cholesterol esters rose in the early, and declined below normal in the later, stages of tumor growth, the regression being quadratic (9). The shifts occurred earlier in hair than in skin. There were no significant shifts in the residual carcass.

There were no significant differences between measurements on males and those on females except that weight of the hair and skin was greater in control males. Loss of weight in skin was greater in male than in female tumor bearers.

Discussion

From a study of shifts in weight and levels of lipids and water in the mesentery of rats bearing Walker carcinosarcoma 256 (2), it was concluded "that shifts in levels of lipids and water in host carcass are (probably) due mostly to shifts in skeletal muscle and connective tissue." The derma and subcutaneous tissue of skin contain large amounts of connective tissue. Lipid and water levels of normal skin are similar to those of normal mesentery in rats of the age used in this study (2).

Shifts in the skin of tumor-bearing rats may be compared, therefore, with those previously reported in mesentery (2) with the reservation that while

conditions were similar they were not identical in the two studies. Reported mean changes are as follows:

	In mesentery, %	In skin, %
Gram fresh weight	− 38	−39
Water	+182	+14
Neutral fat	− 47	−62
Phospholipid	+106	−21
Free cholesterol	+ 92	+40

Taking into consideration the factors behind calculation of each mean, these results indicate that loss of weight and neutral fat were at the same rate in skin as in mesentery, while levels of water and free cholesterol were increased to a lesser extent in skin than in mesentery.

A striking difference from mesentery was the fall in levels of skin phospholipid. In no other organ or tissue of albino rats bearing Walker carcinosarcoma 256 has a statistically significant fall in levels of phospholipid been reported.

If it be assumed that shifts in the connective tissue of skin are the same as those in mesentery, differences noted above must have been due to shifts in the epidermis. This assumption would indicate that in epidermis of the tumor-bearing rat there occurred a marked decline in levels of phospholipid, and probably a fall in the levels of water and free cholesterol. It might be assumed further that these shifts would have been reflected to some extent in the composition of hair.

The composition of hair did not so change, however. The outstanding shift in hair was an increase in the levels of cholesterol esters early in tumor growth followed by a fall to subnormal levels in late tumor growth. This quadratic shift preceded, rather than followed, a corresponding shift in skin. The cholesterol ester fraction, as estimated, included delta-7-cholestenol, which is present in large amounts in rat skin (10).

Following application of methylcholanthrene to the skin of rodents, there follows a decrease in the level of neutral fat, phospholipid, and sterols, especially delta-7-cholestenol (1). The effect of large Walker carcinosarcomata 256 was, therefore, similar to the effect of the carcinogenic agent methylcholanthrene.

The declining levels of skin phospholipid may have significance in the mechanism of production of the sallow appearance of the skin in late malignancy. Declining levels of phospholipid have been found associated with declining physiological activity of an organ (11, 12). Declining levels of phospholipid, along with declining production of hair, may be taken to indicate declining function of the skin. It is probable from, though not proved by, the evidence obtained in this study that decline in phospholipid levels occurred mostly in epidermis.

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LACK OF EFFECT OF THYMECTOMY ON INGUINAL LYMPH GLAND WEIGHT AND THYROID FUNCTION IN WEANLING RATS[1]

E. O. HÖHN

Abstract

Thymectomy (complete in two-thirds of the animals) was performed on 42 weanling rats and a sham operation on 44 controls. Examination of groups of the animals at 1 to 3½ weeks after thymectomy showed no significant differences in inguinal gland weight, thyroid weight, or thyroidal 24-hour uptake of radioactive iodine between the thymectomized and control animals.

There are a number of reasons (e.g. similarity of microscopic structure and of response to hormones) for regarding the thymus as functionally part of the system of lymphoid tissue. If this is the case one would expect that removal of the relatively large thymus of immature animals would be followed by a compensatory hypertrophy of lymphoid tissue. An experiment of this type has been conducted by Gyllensten (1) on newborn guinea pigs. He found a small but significant increase in lymph gland weights 2 weeks following thymectomy, whereas 4 weeks after the operation lymph gland weights of operated and control animals were not significantly different. The only information on the effect of thymectomy in rats seems to be the statement by Pappenheimer (2) that thymectomized rats showed a marked hyperplasia of regional lymph glands 30 to 58 days after thymectomy, but no quantitative data are given. It was therefore decided to re-examine the effect of thymectomy on lymph gland weights in rats. Since Gyllensten (1) had found that the temporary lymph gland hyperplasia after thymectomy affected all regional lymph glands to the same extent, it seemed justified to weigh only the inguinal lymph glands as an indication of changes in the lymph gland weight in general.

Comsa in several publications (see, for example (3)) has described an antithyroid action of thymus extracts, which it is suggested contain a thymus hormone. If the thymus secretes a thyroid-inhibiting hormone thymectomy should be followed by some indications of increased thyroid action and for this reason thyroid weights and the 24-hour uptake of radioactive iodine were also determined in some of the thymectomized rats.

Material and Methods

Female Sprague–Dawley rats weighing initially between 40 and 50 g were used for all the experiments. Thymectomy was performed under ether anaesthesia following the technique of Segaloff (4) on 42 rats. A sham operation was performed on 44 control animals in which the thymus was merely exposed by splitting the manubrium sterni as in the experimental animals, after which the chest wall and neck were sewn up. Groups of thymectomized and control animals were injected intraperitoneally with radioactive iodine,

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Contribution from the Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta.

5 μc of I^{131}, given as sodium iodide in 0.5 ml water. Twenty-four hours later the anaesthetized rats were held on a plastic plate set over the scintillator of an "Atomic" counter attached to a Tracerlab "Superscaler", with the larynx centered over the crystal. Two 1-minute counts were made for each rat. Count rates were expressed as percentages of the count rate given at the same time by a standard sample of the original dose of I^{131} injected, using the same geometry. Such determinations of 24-hour uptake of radioactive iodine were made at 1, 2, and 3 weeks following thymectomy.

At 1, 2, 3, and 3½ weeks following thymectomy groups of thymectomized and control rats were killed with chloroform and weighed. The thyroids of the animals killed 1 and 3 weeks after thymectomy and the inguinal lymph glands of one side of all animals were removed by dissection and preserved overnight in 10% formol saline; they were weighed later, after removal of extraneous moisture by blotting on filter paper, at greater leisure. Macroscopic examination showed regenerated thymus remains in about one-third of the thymectomized animals; in the rest thymus removal had apparently been complete.

Results and Discussion

The results are summarized in Table I.

TABLE I

The effect of thymectomy on weight of inguinal lymph glands of one side, thyroid weight, and 24-hour thyroid uptake of radioactive iodine in weanling rats

Interval between thymectomy and examination, weeks		Inguinal lymph gland, wt. mg/100 g body wt. (mean ± S.E.)	Thyroid wt., mg/100 g body wt. (mean ± S.E.)	% of dose of I^{131} injected in thyroid at 24 hours (mean ± S.E.)
1	Thymectomized	(10) 9.14±1.04	(10) 6.82±0.45	(10) 10.46±1.10
	Controls	(9) 9.36±1.24	(9) 6.72±0.64	(9) 10.56±0.70
2	Thymectomized	(18) 6.70±0.48	—	(10) 12.2 ±0.61
	Controls	(21) 5.65±0.40	—	(10) 12.78±0.40
3	Thymectomized	(8) 5.60±0.76	(8) 9.38±0.70	(8) 12.1 ±0.85
	Controls	(8) 5.70±0.64	(8) 8.36±0.54	(8) 10.6 ±0.76
3½	Thymectomized	(6) 6.90±0.69	—	—
	Controls	(6) 5.40±0.73	—	—

NOTE: The figures in parentheses indicate the number of animals used for any particular measurement.

As regards weight of the inguinal lymph glands there is evidence of slight hyperplasia only in the animals killed at 2 and at 3½ weeks after thymectomy. However, in both cases the differences in mean inguinal lymph gland weight relative to body weight between the operated and control animals were not statistically significant; application of the "t" test gives a "p" value of approximately 0.1 in both cases.

Thyroid weights at 1 and 3 weeks after thymectomy were not significantly different in control and thymectomized animals. Twenty-four hour uptake of radioactive iodine was almost identical in control and thymectomized animals

at 1 and 2 weeks after thymectomy. Three weeks after the operation iodine uptake of thymectomized animals was somewhat greater than in the controls but the difference was not statistically significant, "p" value 0.2.

The experiments therefore failed to confirm Pappenheimer's (2) statement that thymectomy in the rat produces a marked hyperplasia of lymph glands, at any rate as far as the inguinal lymph glands are concerned. The absence of any indications of increased thyroid function from 1 to 3 weeks following thymectomy is in agreement with the recent report on the effect of thymectomy on thyroid function in weanling rats of the hooded Lister strain by Doniach (5). In his experiment thyroid weights were determined 10 days after thymectomy and uptake of radioactive iodine was determined 2 days after thymectomy and its release was followed by daily measurements for the next 7 days. No difference in these values for thymectomized and control animals was found. Most of the experiments reviewed by Gyllensten (1) also show either no effect or only slight stimulation of thyroid function following thymectomy though his own experiments on guinea pigs show a significant increase in thyroid weight and oxygen consumption 2 weeks following thymectomy.

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ZONE ELECTROPHORESIS OF THE PROTEINS OF THE FOWL'S SERUM AND EGG YOLK[1]

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Abstract

In addition to the six protein fractions distinguishable in sera from cocks or sexually immature pullets by zone electrophoresis in aqueous veronal, two lipoprotein zones have now been distinguished in sera from laying hens or estrogenized pullets by this same technique. Phosvitin has been detected in serum of estrogenized hens by zone electrophoresis of a saline solution of crude serum lipovitellin obtained from the serum. Six protein zones have been distinguished in similar electropherograms of egg yolk. The six protein zones have been identified provisionally, and in order of decreasing mobility under the conditions used, as phosvitin, α-livetin, β-livetin, γ-livetin, lipoprotein P-1 (possibly a lipovitellenin complex), and lipoprotein P-2 (possibly a lipovitellin complex). Evidence is submitted that the foregoing two major lipoprotein zones from egg yolk are closely similar to, or identical with, the two lipoprotein zones from sera of laying hens or estrogenized pullets. The status of a seventh minor protein zone in electropherograms of yolk protein is uncertain.

Introduction

Six protein fractions have been distinguished in the serum of the cock or sexually immature pullet by zone electrophoresis on paper (1) when the electrophoresis is performed in aqueous veronal buffer, at pH 8.6, by the technique of Flynn and DeMayo (2). These six zones have been designated in order of decreasing mobility, at pH 8.6, as follows: albumin, α_1-globulin, α_2-globulin, α_3-globulin, β-globulin, and γ-globulin. These six zones have been distinguished also in buffer made up in the same proportions but with 20% v/v methanol instead of water (1, 3). Earlier studies with sera from laying hens or from estrogenized fowl had shown an additional lipid-rich zone that moved slowly in aqueous buffer at pH 8.6 (4). This zone was designated provisionally as "presumptive lipovitellin" (PLV). It proved difficult at that time to distinguish this PLV zone from other zones or to resolve it further when the buffer was aqueous veronal, but use of veronal buffer made up with 20% methanol enabled two lipoprotein zones to be distinguished clearly on the resultant electropherograms (5). The slower of these zones, under the conditions of electrophoresis used, was apparently rich in protein phosphorus (1) and was designated as zone PP. The faster lipoprotein zone, which was also the wider of the two, was designated fraction 8 by Vanstone *et al.* (3). These workers also used methanolic veronal in their study of the changes in serum proteins from the stage of the 14-day embryo to that of full reproductive activity (3).

McKinley *et al.* (1) applied the methanolic buffer technique to the examination of the proteins of egg yolk. The electropherograms showed two lipoprotein zones, of which one evidently corresponded to the PP zone of the serum protein

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Contribution from the Faculty of Agriculture of McGill University, Macdonald College, Macdonald College P.O., Que.
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of laying hens, while the other corresponded to the second lipoprotein zone (fraction 8) of such sera. The electropherograms also showed two lipid-free zones, X and Y, that represented non-vitellin components of yolk.

The foregoing studies were based on the use of apparatus constructed and operated according to the directions of Flynn and DeMayo (2) and most of the observations were made with use of the methanolic buffer. However, although methanolic buffer had proved useful in the original separation of two lipoprotein zones, it seemed likely that its use must involve undue complexity of the conditions of electrophoresis, e.g., through differential evaporation. It was obviously desirable, if possible, to resolve the lipoproteins in aqueous buffer. In addition, it was decided to study the suitability of more compact electrophoretic cells because of the heavy expenditure of buffer involved in the use of the original large cells.

The present paper reports (*a*) the successful resolution of the lipoprotein (PLV) fraction of serum into two lipoprotein zones in aqueous veronal; (*b*) a comparison of resolutions obtained in a Matthew electrophoresis cell (similar in principle to the cell described by Flynn and DeMayo but more compact) with those obtained in a standard Spinco–Durrum cell of the type described by Durrum (6); and (*c*) further observations on the resolution of egg-yolk proteins by zone electrophoresis, including some preliminary quantitative observations.

Experimental Methods

Apparatus

(1) The Matthew cell (Matthew Laboratories, New York, N.Y.) was simplified in operation by supporting the paper (Whatman 3 MM) on a sheet of pebbled "plexiglas" (size P4) instead of on the supporting racks supplied by the manufacturer (7). "Plexiglas" supports have been found completely satisfactory. Runs were made at constant amperage of 0.57 milliampere per inch width of paper.

(2) The Spinco–Durrum cell (Spinco Model R) was used according to the instructions of the manufacturer (Beckman Instruments Inc., Belmont, California) without modification. Runs were made at a constant amperage of 0.60 milliampere per inch width of paper.

Buffer

Veronal buffer was prepared by dissolving 1.84 g diethylbarbituric acid and 10.30 g sodium diethylbarbiturate in 1.0 liter distilled water.

Staining

(1) Strips cut from the electropherograms for protein staining were air-dried and left overnight in ethanol–ether (3:1 v/v) acidified with 5% trichloroacetic acid. Next morning the solvent was warmed and then poured off. The strips were next refluxed for three successive ½-hour periods with ethanol–chloroform (1:1 v/v) containing 5% trichloroacetic acid. The papers were stained by the method described by Kawerau (8), except that no purification of the Amidoschwarz was required. This stain was that manufactured by Chromagesellschaft (Stuttgart, Germany).

(2) Strips cut from the electropherograms were stained for lipid with oil red O as described by Durrum *et al.* (9).

Blood Sampling

Blood samples were drawn from the wing vein through a No. 18 needle and allowed to clot spontaneously. The serum was separated by centrifugation. Sera were kept at 5° C for not longer than 2 days before electrophoresis. We have noted, however, that it is possible to obtain satisfactory electropherograms of samples of cock serum that have been kept for as long as 2 weeks at 5° C. Serum from estrogenized fowl may also be kept for some days at 5° C without alteration of the zone electrophoretic pattern.

Experimental Results

1. Comparison of Sera from the Cock, from the Laying Hen, and from the Estrogenized Non-laying Hen by Use of the Matthew Cell

The sera were run in aqueous veronal buffer with results exemplified by Fig. 1. Good resolution of the usual six zones was secured for the cock's serum (strip A). The α_1-globulin was reasonably well distinguished. Lipid staining was present slightly ahead of the α_1-globulin zone, as has been observed previously.

The results for the laying hens' sera (Fig. 1, strip B) were satisfactory. The α_2-, α_3-, β-, and γ-globulin zones were well defined, but an α_1-globulin zone was not clearly resolved nor was there any lipid staining in this region. The latter observation confirms previous similar observations on sera of laying hens and estrogenized pullets. The most striking result was the satisfactory resolution of two distinct lipoprotein zones in aqueous buffer. These will be designated P-1 and P-2 (Fig. 1, strips B and C), for purposes of the present paper. "Channelling" and confusion of the zones in this region hampered earlier studies (4) based on the use of aqueous buffer, but these troubles were avoided by the technique used in the present studies.

The results with sera of the estrogenized birds (Fig. 1, strip C), in which the serum phosphoprotein level was high, were equally satisfactory. It is possible that the P-2 zone corresponds with the PP zone of Vanstone *et al.* (3) and the P-1 zone with the fraction 8 described by the same authors. Zones P-1 and P-2 also appear to correspond with the two major peaks described by Evans and Bandemer (10) for electropherograms of egg yolk run in aqueous buffer. The relation of the two zones to the X_1 and X_2 components distinguished in the ultracentrifuge by Scheijde and Urist (11) is less obvious, although their fraction X_1 may be related to the P-2 fraction now discussed.

None of the foregoing runs provided any evidence of the presence of phosvitin, although phosvitin has recently been isolated from the serum of the estrogenized laying hen (12) and of the laying hen (unpublished observation in this laboratory). Its presence has been demonstrated by zone electrophoresis as follows. Serum from heavily estrogenized birds was diluted 10-fold with distilled water and the crude lipovitellin complex was separated on the centrifuge and redissolved in 0.15 *M* NaCl. This solution was then treated with sufficient oxalate

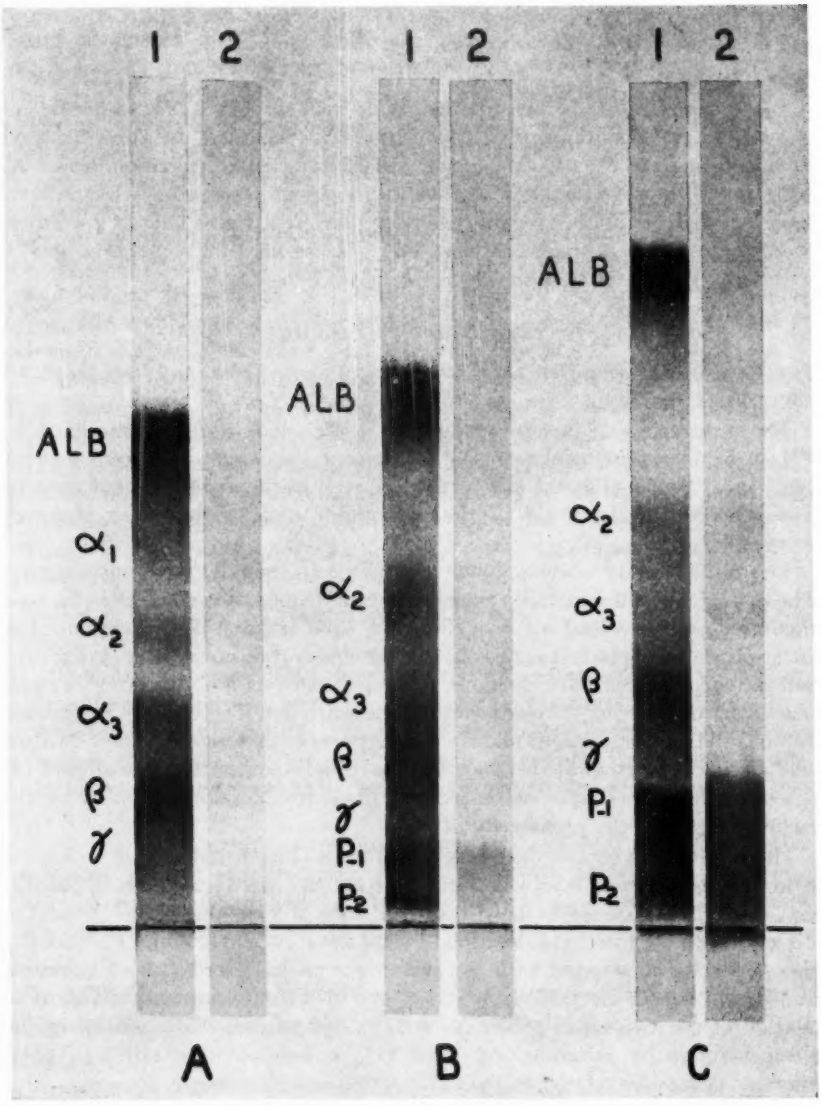


FIG. 1. Separation in aqueous buffer of protein zones of sera from cock, laying hen, and estrogenized non-laying hen.

(A) Cock's serum: Matthew cell with veronal buffer, pH 8.6; $\mu = 0.05$. Temp. 5° C. Stains: (1) Amidoschwarz (protein) and (2) oil red O (lipid). Sample applied at cathodic end of paper.

(B) Laying hen's serum: conditions as for strip A.

(C) Estrogenized non-laying hen's serum: conditions as for strip A.

to precipitate calcium. The precipitate was removed next morning by centrifugation and the supernatant was subjected to zone electrophoresis as described above. The electropherograms showed (*a*) no indication of albumin or of α_1-, α_2-, or α_3-globulins and (*b*) the presence of a lipoprotein zone corresponding in position with P-1 and a broad protein zone in advance thereof. These latter two fractions may be related to the two centrifugal fractions of the serum lipophosphoprotein complex mentioned by McIndoe (13). In addition there was a very faintly staining band which had a mobility, under the given conditions, greater than that of serum albumin and the same as that of a sample of egg yolk phosvitin run concurrently. We have not succeeded, however, in demonstrating the presence of phosvitin in serum of the fowl by zone electrophoresis of untreated serum.

The major conclusions from the foregoing experiments are (*a*) that two lipophosphoprotein components can be distinguished in electropherograms of sera from laying or estrogenized birds by direct zone electrophoresis in aqueous veronal buffer in a cell of the Matthew type; (*b*) that zone electrophoresis of a solution of the crude lipovitellin complex (obtained by dissolving crude serum lipovitellin complex in 0.15 *M* NaCl, and subsequent removal of calcium ions by addition of oxalate and centrifugation) yields a lipoprotein zone, a second wider lipid-free zone, and a very faintly staining phosvitin zone.

2. Effect of Position of Application of Sample on Resolution and a Comparison of Results with the Matthew Cell and with the Spinco–Durrum Cell

Cock's serum was applied to strips in the Matthew cell (*a*) in the normal position near the cathodic end (Fig. 2, strip D) and (*b*) midway between the cathodic and anodic ends (Fig. 2, strip E). A sample of the same serum was run simultaneously in the Spinco–Durrum cell with the same buffer and by the technique ordinarily used with this cell (Fig. 2, strip F).

The resolution of the β- and γ-globulin fractions was less distinct on strip E than on strip D. The results with strip F (Spinco–Durrum cell) showed narrow, well-defined zones for α_1-, α_2-, and α_3-globulins, but the resolution of β- and γ-globulins was relatively poor.

Similar comparisons were made for samples of serum from laying hens (Fig. 2, strips G, H, and I). The results show that the resolutions by the Spinco–Durrum technique or by the Matthew cell technique with application midway between the electrodes were inferior to that secured by the Matthew cell with application near the cathodic end. Accordingly, the latter technique was adopted for all the electropherograms discussed below.

3. Zone Electrophoresis of Egg Yolk Proteins

As already mentioned above, it was considered that the P-1 and P-2 components noted in the sera of laying and estrogenized fowl might correspond broadly with the two major lipoprotein fractions noted in such sera by McKinley *et al.* (5) and by Vanstone *et al.* (3) and in egg yolk by McKinley *et al.* (5), and even more clearly with the two major lipoprotein peaks noted by Evans and Bandemer (10) in electropherograms of yolk in view of the latter workers' use of aqueous buffer. In order to study this point further, yolks from freshly

laid eggs were snipped clear from the chalazae and freed from adherent egg white by rolling the yolks on a large filter paper. A sample (2 ml) was withdrawn from each yolk by hypodermic syringe and diluted with 5 ml 1% NaCl. The mixture was centrifuged. The supernatant was reserved for separate examination (see below). The deposit was dissolved in 10% NaCl and made up to a volume of 7 ml with the same solution of NaCl.

Electropherograms were run on (*a*) the whole yolk diluted with 1% NaCl (Fig. 3, strip J); (*b*) a sample of α- plus β-livetins prepared by free electrophoresis and containing traces of γ-livetin* (Fig. 3, strip K); (*c*) the supernatant from centrifugation of the diluted egg yolk (Fig. 3, strip L); and (*d*) the deposit from centrifugation of the diluted egg yolk (Fig. 3, strip M).

Strip J (diluted egg yolk) showed clearly two lipoprotein zones which have been designated P-1 and P-2 on account of their correspondence with the P-1 and P-2 bands of the sera of laying or estrogenized fowl. We have identified zone P-1 tentatively as lipovitellenin or, more probably, a complex thereof; and P-2, which we believe corresponds with the PP fraction noted by others (1, 3), as lipovitellin or, rather, 'lipovitellin complex' (14). Two prominent lipid-free protein zones were obviously identical with the X and Y zones of yolk proteins noted by McKinley *et al.* (1). A third less intensive zone was noted and was designated zone Z. On referring to strip K, which was run in parallel with strip J, it will be noted that zone X corresponded with the α-livetin of Martin *et al.* (15, 16) and zone Y corresponded with the β-livetin of the same workers. Zone Z likewise corresponded with a third faint band in the livetin preparation. Zone Z may be identified, therefore, with γ-livetin (16), since it was known that the preparation contained traces of the latter protein.

A point of major interest in connection with strip J was the presence of a faintly staining but distinct zone that ran well in advance of the α-livetin zone. The position of this zone and the faintness of its staining with Amidoschwarz corresponded with the position and staining properties of phosvitin preparations isolated from egg yolk, as will be shown below. This zone by no means necessarily represents all of the phosvitin of the yolk. It may merely represent a part of the phosvitin that is readily liberated from 'lipovitellin complex' (14) under the conditions of zone electrophoresis. The P-1 fraction appeared not to liberate phosvitin under these conditions. A further point of possible practical significance is that the phosvitin zone was more distinct when the dilution of egg yolk had been left to stand in the cold at 5° C for 2 or 3 days.

*This material was kindly presented by Dr. W. H. Cook, Division of Applied Biology National Research Council, Ottawa.

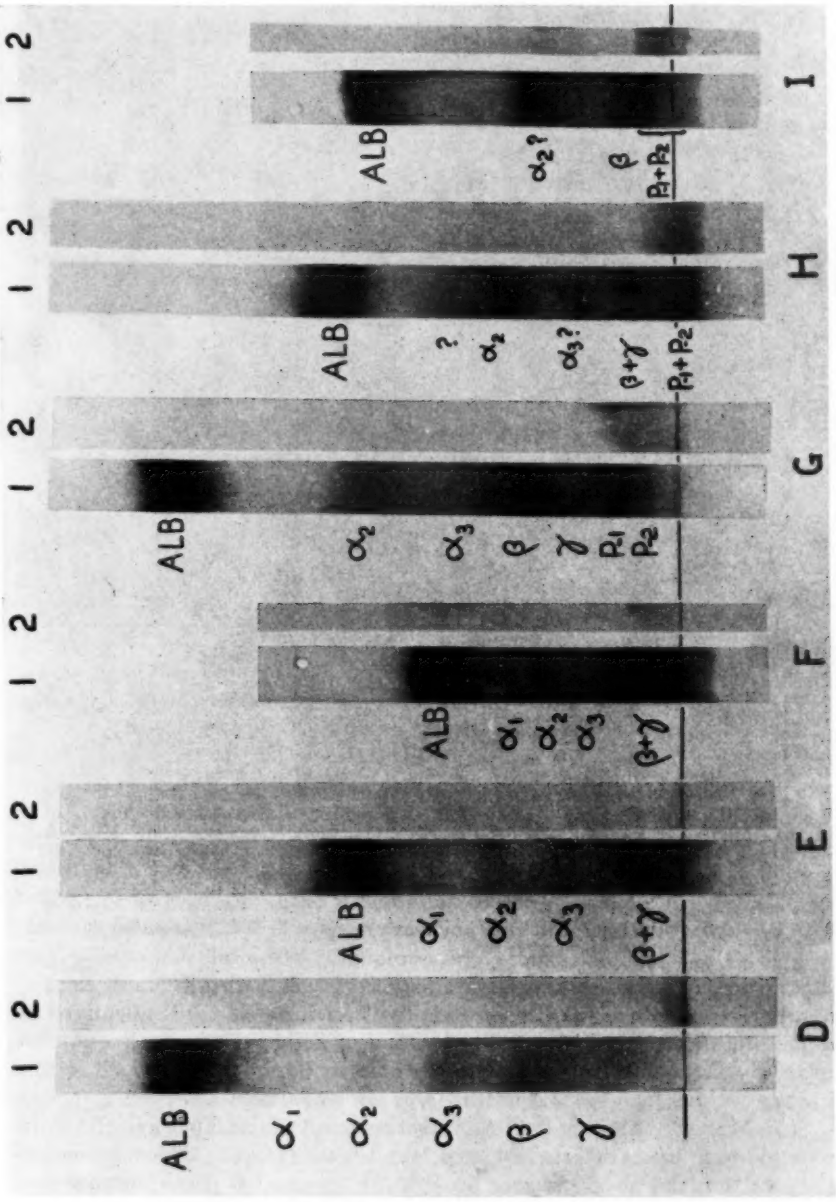
FIG. 2 (see p. 1463). Comparison of results with Matthew cell and Spinco–Durrum electrophoretic cells.

(D) Cock's serum: Matthew cell with veronal buffer, pH 8.6; $\mu = 0.05$. Temp. 5° C. Stains: (1) Amidoschwarz (protein) and (2) oil red O (lipid). Sample applied at cathodic end of strip.

(E) Cock's serum: conditions as for strip A but sample applied midway between cathodic and anodic ends of strip.

(F) Cock's serum: Spinco–Durrum cell with veronal buffer, pH 8.6; $\mu = 0.05$. Application of sample in normal position for Spinco–Durrum operation.

(G, H, I) Laying hen's serum: conditions as for strips D, E, and F respectively.


1 2
ALB
α1
α2
α3
β
γ
D
ALB
α1
α2
α3
β+γ
E
ALB
α2
α3
β
γ
P1
P2
F
ALB
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α2
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β+γ
P1+P2
G
ALB
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P1+P2
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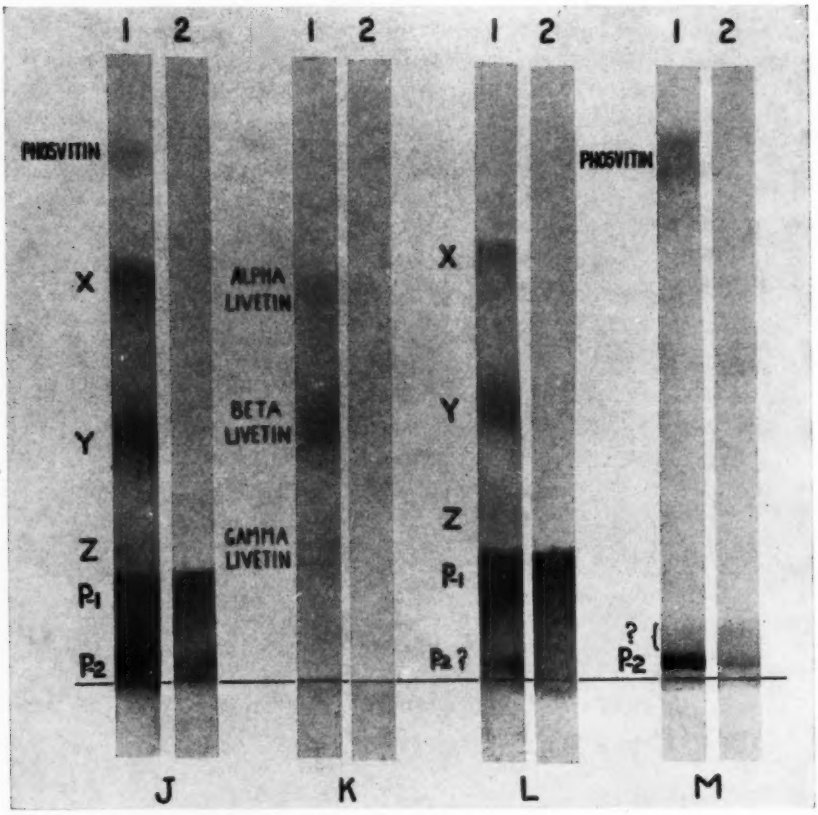

FIG. 3. Zone electrophoresis of egg yolk proteins.

(J) Whole egg yolk suspended in 1% NaCl. Matthew cell with veronal buffer, pH 8.6; $\mu = 0.05$. Temp. 5° C. Stains: (1) Amidoschwarz (protein) and (2) oil red O (lipid).

(K) Mixture of livetins isolated by free electrophoresis (courtesy Dr. W. H. Cook, National Research Council, Ottawa) dissolved in veronal buffer. Other conditions as for strip J.

(L) Supernatant from centrifugation of suspension of egg yolk in 1% NaCl. Other conditions as for strip J.

(M) Deposit from centrifugation of suspension of egg yolk in 1% NaCl, dissolved in 10% NaCl. Other conditions as for strip J.

Strip L (supernatant from the dilution of egg yolk with 1% NaCl) showed clearly the presence of P-1 (the presumptive lipovitellenin), of X (identified as α-livetin), of Y (identified as β-livetin), and of Z (identified as γ-livetin). A relatively slight P-2 zone was probably due to incomplete removal of this fraction, since a high-speed centrifuge was not available.

Strip M showed a strong P-2 lipoprotein zone and a faint, lipid-free phosvitin zone. Phosvitin was detected on strip M but not on strip L. This observation suggests that the phosvitin zone on strip M represented phosvitin that was originally associated with the material that settled on centrifugation of the diluted egg yolk, but that dissociated readily from this material under the

conditions of zone electrophoresis. Joubert and Cook (14) considered that phosvitin and γ-livetin are associated with lipovitellin to form a "lipovitellin complex", but that phosvitin is not associated with lipovitellenin. The circumstance that phosvitin appeared on the electropherogram in which zone P-2 is present but not in those from which P-2 is absent might be thought, therefore, to point to the identification of P-2 as lipovitellin (or lipovitellin+ γ-livetin) and of P-1 as lipovitellenin. However, as will be shown below, the N:P ratios of zones P-1 and P-2 were very similar, so that it is not yet possible categorically to identify P-1 with lipovitellenin and P-2 with lipovitellin. Both zones were probably complexes, and both may well have contained phosvitin even though P-2 obviously can be separated at least in part from phosvitin by zone electrophoresis.

Strip M also showed the presence of yet another zone which lay just ahead of the P-2 zone and which is marked with an interrogation mark in Fig. 3, strip M. This zone stained faintly with Amidoschwarz and also with oil red O.

The foregoing experiments have shown that it is possible readily to distinguish six protein fractions of egg yolk by zone electrophoresis in aqueous veronal on paper. One of the zones is phosvitin; three are livetins, viz., α-, β-, and γ-livetin; and two major lipoprotein zones may possibly represent lipovitellenin and lipovitellin complexes. A third minor lipoprotein zone is of uncertain significance.

4. A Comparison of the Phosvitin Zone in Electropherograms of Whole Egg Yolk and of the Separated P-2 Fraction with the Phosvitin Zone Given by Egg Yolk Phosvitin

Egg yolk was diluted with 1% NaCl and then run as described above (strip N). Lipovitellin complex was separated by dilution of 2 volumes of yolk with 5 volumes of 1% NaCl and centrifugation; the deposit was dissolved in 10% NaCl and this solution was run in parallel with the whole diluted egg yolk to give strip P. A sample of phosvitin that had been prepared from egg yolk by the method of Joubert and Cook (17) was dissolved in 10% NaCl and run in parallel with the foregoing two strips to yield strip O. The results are presented in Fig. 4.

Strip N showed the usual six protein zones including a faintly staining phosvitin zone. Strip P showed the usual P-2 lipoprotein zone and a phosvitin zone. There was also a slight lipoprotein zone immediately in advance of the P-2 zone, similar to that shown on strip M. Strip O showed clearly a faintly staining phosvitin zone which had the same mobility as the phosvitin of strip P under the given conditions.

Strip O (egg yolk phosvitin preparation) gave a leading faint zone which moved at the same rate under the given conditions as the similar zones on strips N and P. (This also confirmed the identification of the phosvitin zones on strips J and M.) The phosvitin on strip O also gave a second (and possibly two) slower zones. However, it is known that phosvitin forms complexes depending on the solvent and that it may then run as two or three zones or as a single very broad one (Dr. W. H. Cook, private communication). The ease

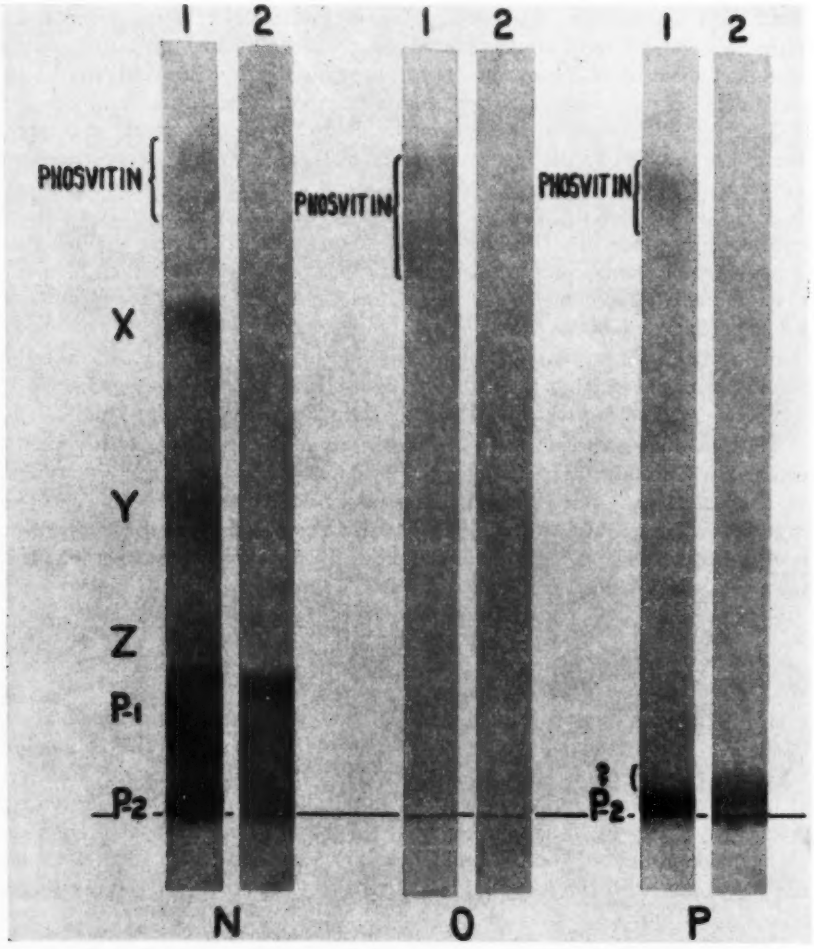

FIG. 4. Comparison of movement of presumptive phosvitin band of egg yolk suspension in 1% NaCl with that of phosvitin prepared from egg yolk. Matthew cell with veronal buffer, pH 8.6; $\mu = 0.05$. Temp. 5° C. Stains: (1) Amidoschwarz (protein) and (2) oil red O (lipid).
(N) Egg yolk suspension in 1% NaCl.
(O) Phosvitin (egg yolk) dissolved in 10% NaCl.
(P) Fraction P-2 in 10% NaCl.

with which Amidoschwarz, azocarmine B, or bromophenol blue are washed out from stained phosvitin zones complicates the study of these zones but is useful, at the same time, in their identification. For the same reason, absorptiometric measurements on phosvitin zones are probably of relatively little value for purposes of estimating the amounts of phosvitin.

5. The Proportions of Fractions P-1 and P-2 in Egg Yolk

Egg yolk was diluted with 1% NaCl (2:5 v/v) and the dilution was applied to sheets of Whatman 3 MM paper at the rate of 200 microliters per 3.5-in. width of paper. Electrophoresis was carried out in the Matthew cell as described above. Lipid was extracted from the papers, and the protein zones were located by staining strips cut from the edges. The zones corresponding to fractions P-1 and P-2 were then cut out and analyzed for N by the micro-Kjeldahl method and for P by the method of Martin and Doty (18) after digestion of the paper with nitric and perchloric acids. Appropriate blank corrections were applied. The results are presented in Table I.

TABLE I

Proportions of fractions P-1 and P-2 in egg yolk protein

Constituent	Fraction P-1	Fraction P-2
Protein N, mg per ml yolk	6.70	10.74
Protein N, % of total yolk N	24.4	39.1
Protein P, mg per ml yolk	0.45	0.68
Ratio, protein N: protein P	14.9	15.8

Two facts emerge from these preliminary analytical data. Firstly, the lipoproteins accounted for only about two-thirds of the total protein N of the yolk. Hence the livetin fractions, together with such phosvitin as becomes dissociated from lipoprotein complexes to migrate in front of the α-livetin, apparently represented about one-third of the total yolk protein N. This is a higher proportion of livetins than has been reported by various workers, but it is in general accord with the results of the sedimentation studies of Martin, Vandegaer, and Cook (15). Secondly, the ratio protein N:protein P was practically the same for both fractions in spite of their electrophoretic separation. This fact, and the size of the ratios, suggest that both P-1 and P-2 may have contained a considerable proportion of phosvitin. Further work on these quantitative aspects of zone electrophoresis of yolk proteins is in progress.

Acknowledgments

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ISOMERIZATION OF VITAMIN A IN VIVO[1]

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Abstract

Pure all-*trans* and neovitamin A alcohol were administered orally to young, vitamin-A-deficient rats and the vitamin A and proportion of neovitamin A in the intestinal tract, liver and kidney were measured. In both cases considerable isomerization occurred in the stomach and the resulting mixture of isomers appeared to be absorbed by the intestine. The dose of all-*trans* vitamin A was taken up by the intestine more quickly than was the neovitamin A dose. An oral or subcutaneous dose of neovitamin A resulted in a relatively high proportion of neovitamin A in the liver but this proportion decreased during depletion to that found after a dose of all-*trans* vitamin A. The proportion of neovitamin A varied inversely with the size of dose of all-*trans* vitamin A and directly with the size of dose of neovitamin A. The significance of these results is discussed.

Vitamin A can exist in one of several isomeric forms, all-*trans*, neovitamin A (13-mono-*cis*), *neo-b* (11-mono-*cis*), 9-mono-*cis*, 11,13-di-*cis*, and 9,13-di-*cis*, which vary widely in their biological activity (1, 2). Neovitamin A has been reported to occur to the extent of about 30% in many fish-liver oils (3, 4, 5) and synthetic sources (6, 7, 8), although Lambertsen and Braekkan (9) doubt its occurrence in vivo. Other *cis* isomers have been found in various crustacea (10, 11, 12), but Morton and Bro-Rasmussen (13) were unable to detect them in fish oils. The fact that the spectrophotometric method (2) has been found to yield a good estimate of biological potency of fish-liver oils is also evidence against the occurrence in quantity of *cis* isomers other than neovitamin A, for these isomers would contribute proportionately much more to the absorbance at 325 mμ than to biological activity. Plack (14) fed the 11-*cis* isomer to rats and found very little of the liver vitamin A stores in this form. Brown (15) was unable to find any evidence of the *neo-b* isomer in cattle liver or serum and in experiments on rats Dowling and Wald (16) obtained results similar to those of Plack.

There is evidence, however, that an equilibrium mixture of isomers is formed in rat livers. Harris, Ames, and Brinkman (17) found that the livers of rats fed all-*trans* or neovitamin A contained approximately the same neovitamin A content, i.e. 11–19%. Preliminary experiments in this laboratory (18) showed that after a dose of all-*trans* or neovitamin A, considerable interconversion occurred in the intestinal tract and the liver with the all-*trans* isomer being the predominant form found. Such data, however, are still incomplete and since neovitamin A is a common constituent of commercial sources of vitamin A activity a more exhaustive study was deemed necessary in order to understand better the metabolism of vitamin A by animals.

It is the purpose of this paper to report on the proportion of isomers found in the various tissues after dosing with all-*trans* or neovitamin A and to attempt to explain the lower biological potency of the latter as compared with all-*trans*.

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[2]N.R.C. Postdoctorate Fellow 1958.

Procedure and Results

Isomer Content of the Various Tissues Following All-trans or Neovitamin A Dosing

Weanling male rats of an inbred Wistar strain were fed the vitamin-A-free diet (2) for 2 weeks, after which time no vitamin A could be detected in the liver, and were then divided into two groups. One group received 4916 units of all-*trans* vitamin A alcohol, the other 2500 units of neovitamin A alcohol* dissolved in corn oil and administered orally with a tuberculin syringe and blunted needle. Five rats from each group were killed by decapitation after intervals of 1, 2, 3, 4, 5, 24, and 48 hours from dosing. The intestinal tract, liver, and kidneys were removed and analyzed for vitamin A and neovitamin A. The stomach and caecum were analyzed with their contents, intestinal contents were expressed manually, and the intestine washed with 0.9% saline. The kidneys from both groups of animals were divided into three fractions: 1–5 hours, 24 hours, and 48 hours. The method of Ames, Risley, and Harris (19) was used to estimate the vitamin A content of the tissues except in the case of the intestinal contents which were saponified prior to extraction with petroleum ether. Neovitamin A was measured by the maleic anhydride method (3), which distinguishes between fast- and slow-reacting isomers, but the results were expressed in terms of the principal isomers in these groups, all-*trans* and neovitamin A. Hayes and Petitpierre (5) found this procedure to have a precision of $\pm 1.5\%$.

The values in Tables I and II are, in most cases, average values for five rats but bulking of tissues was necessary in certain cases to obtain sufficient vitamin A for analysis.

TABLE I

Neovitamin A found in the various tissues following all-*trans* or neovitamin A dosing expressed as percentage of the total vitamin A content

	% neovitamin A found in the tissues, hours after dose													
	2500 units* neovitamin A							4916 units* all-*trans* vitamin A						
Tissue	1	2	3	4	5	24	48	1	2	3	4	5	24	48
Stomach	26	42	40	35	45	34	ND†	25	35	18	20	23	30	ND
Intestine	33	37	44	42	42	26	ND	18	18	20	16	10	27	ND
Intestinal contents	33	23	29	29	32	ND	ND	25	22	11	18	25	ND	ND
Liver	32	18	26	24	23	11	11	12	12	4	5	3	3	4
Caecum	78	40	56	41	36	32	59	58	57	39	25	30	26	58
Kidney			20			25	29			18			13	12

*Determined by $SbCl_3$ reaction.
†Not detectable.

The percentage neovitamin A found in the tissues following the above dosing regimes is shown in Table I. When neovitamin A was given, less than 50% of the vitamin A in the stomach was in this form and a similar mixture of isomers was found in the intestine and intestinal contents throughout the experimental period. The liver contained from 18% to 30% of its vitamin A as *neo* for the first 5 hours but within 24 hours the *cis* isomer content had significantly

*Kindly donated by Distillation Products Industries, Rochester, N.Y., through the courtesy of Dr. S. R. Ames.

TABLE II

Vitamin A distribution in the tissues following all-*trans* or neovitamin A dosing

	Vitamin A stores expressed as % of the dose, hours after dose													
	2500 units* neovitamin A							4916 units* all-*trans* vitamin A						
Tissue	1	2	3	4	5	24	48	1	2	3	4	5	24	48
Stomach	36	43	30	15	19	0.7	ND†	48	38	15	14	14	0.2	ND
Intestine	3	3	5	4	5	2	ND	7	6	10	5	4	2	ND
Intestinal contents	14	10	22	15	5	ND	ND	17	13	15	3	4	ND	ND
Liver	2	2	8	11	12	30	36	4	8	10	16	20	32	49
Caecum	0.2	1	0.2	1	7	3	2	0.2	0.2	0.4	4	7	2	0.6
Kidney ($SbCl_3$ units)						4.8	3.2						6.5	4.5
			2.7							3.8				
% recovery	55	59	65	46	48	36	41	76	65	50	42	49	36	53

*Determined by $SbCl_3$ reaction.
†Not detectable.

dropped to 11% ($P = 0.05$). Some isomerization of the all-*trans* vitamin A dose also occurred in the stomach but the all-*trans* form predominated and a smaller proportion of the *cis* isomer was found than when neovitamin A was fed. The liver initially contained 12% neovitamin A but this quickly dropped ($P = 0.01$) to about 4% and changed little thereafter. Compared to this reduction, kidney and caecum neovitamin A remained high ever after 48 hours, although somewhat less *cis* isomer was found in the kidneys of all-*trans* dosed animals. The absolute amounts of *cis* isomer found in these tissues were, however, very low.

The actual vitamin A distribution in the tissues differed with *neo* and all-*trans* dosing as shown in Table II. When all-*trans* vitamin A was fed, significantly more of the dose was found in the intestinal walls ($P = 0.01$) during the first 3 hours. Liver stores also appeared to increase more rapidly after an all-*trans* dose but the difference was not significant. No differences were noticed in the kidney or caecum vitamin A levels following either all-*trans* or neovitamin A dosing.

Vitamin A Depletion Studies

Weanling, vitamin-A-deficient rats were dosed orally or subcutaneously with a water-soluble dispersion of either *neo* or all-*trans* vitamin A, prepared immediately before use in the following manner. The vitamin A and 50 mg α-tocopherol were dissolved in a few drops of ethyl ether and 2 g of Tween 80 was added. After mixing, the ether was removed under vacuum and the mixture warmed gently to 50° C with stirring. A solution of 0.1% ascorbic acid was then added, with shaking, to make a final volume of 8 ml. In a further experiment vitamin-A-deficient animals were injected subcutaneously with a commercial preparation containing 70% all-*trans* and 30% neovitamin A, while another group was given a similar dose of all-*trans* vitamin A. In both experiments five rats from each group were killed after various time intervals, the livers and kidneys removed and analyzed for total vitamin A stores and isomer content.

The results are shown in Table III. Vitamin A stores in the liver progressively decreased with time except that after subcutaneous injection there was a delay before maximum storage was attained. The percentage neovitamin A in

TABLE III

Vitamin A and neovitamin A content of liver and kidney during depletion

		Days after dosing							
		2		7		21		35	
Tissue	Dose	Vit. A, units*	Neo, %	Vit. A, units*	Neo, %	Vit. A, units*	Neo, %	Vit. A, units*	Neo, %
(1) Liver	All-*trans* (920 units* orally)	317	5.2	264	4.2	132	5.8	67	6.4
(2) Liver	All-*trans* (920 units* subcutaneously)	262	4.9	293	3.8	217	1.9	76	4.9
(3) Liver	*Neo* (860 units* orally)	227	10.6	202	8.8	98	4.8	28	3.3
(4) Liver	*Neo* (860 units* subcutaneously)	243	14.9	222	4.1	118	5.5	38	5.3
(5) Kidney	All-*trans* (1 and 2 bulked)	1.8	18.9	2.1	27.4	2.5	14.9	3.4	3.3
(6) Kidney	*Neo* (3 and 4 bulked)	2.6	14.2	2.4	16.1	2.6	29.7	2.4	6.1
(7) Liver	All-*trans* (1250 units* subcutaneously)	430	3.1	535	5.5	500	2.3	—	—
(8) Liver	30% *neo* (1250 units* subcutaneously)	495	12.0	565	7.7	470	2.8	—	—

*Determined by $SbCl_3$ reaction.

the liver after a dose of all-*trans* did not alter appreciably with time, but after administration of either neovitamin A or the 30% *neo* preparation, the proportion of *cis* isomer found in the liver quickly dropped ($P = 0.01$) from an initial high value to that found after all-*trans* had been fed. Following a dose of either all-*trans* or neovitamin A the kidney contained a relatively large proportion of neovitamin A, but in both cases the percentage neovitamin A had decreased by the end of the depletion period. Because only small amounts of vitamin A are stored in the kidney it was necessary to bulk these organs and statistical analysis of the results was not possible.

Effect of Dosage Level on Isomer Composition of the Liver

Corn oil dilutions of all-*trans* or neovitamin A were given orally in single doses ranging from 4900 I.U. to 300 I.U. to weanling, male, vitamin-A-deficient rats. Animals were killed after 48 hours, the livers removed and analyzed for total vitamin A and isomer content.

The results are presented in Table IV. With all-*trans* dosing the percentage of *neo* stored varied inversely with the dose, high doses producing low propor-

TABLE IV

Comparison of liver vitamin A following graded doses of *neo* and all-*trans* vitamin A (after 48 hours)

	Neo dose, units*				All-*trans* dose, units*			
	2500	1250	625	313	4916	2458	1229	615
Vitamin A, units*/liver	912	426	166	85	2759	1340	607	304
Neovitamin A, %	19.4	12.3	7.7	10.3	0.8	1.9	2.9	5.8

*Determined by $SbCl_3$ reaction.

tions of *cis* isomers in the liver ($P = 0.01$). The percentage neovitamin A found did not exceed the relatively low value of 5.8%. Following neovitamin A intake a direct relationship was found between dosage level and proportion of neovitamin A in the liver ($P = 0.05$).

Discussion

It has been shown that in the 24 hours following an oral dose of all-*trans* vitamin A the proportion of neovitamin A in the stomach varied from 18% to

35%. Individual rats showed great variability in this regard but there was no trend toward higher or lower values with time. A similar mixture was found in the intestinal contents and walls and under these conditions maximum storage was obtained in the liver. When neovitamin A was given, the proportion of neovitamin A in the stomach varied from 26% to 45% and while the intestine absorbed this mixture it did not do so as efficiently and liver stores smaller than after an all-*trans* dose resulted.

Early in the experiment the proportion of neovitamin A in the liver resembled that found in the intestine, but this proportion dropped within a few hours, suggesting that isomerization occurred in the liver. This observation was supported by later experiments. Apart from the initial change in the stomach no reduction in neovitamin A was observed in any part of the intestinal tract.

Over the first 3 hours more vitamin A was found in the intestinal walls following all-*trans* dosing than after administration of neovitamin A. Although the difference was not significant it is also worthy of mention that during the first 5 hours after dosing with all-*trans*, the liver stores reached 41% of their final (48-hour) value whereas in the same interval a neovitamin A dose produced only 33% of the final liver stores. Dowling and Wald (16) observed a similar delay in the action of the 11-*cis* isomer in the curing of night blindness in the rat.

If the intestine absorbed less of a dose of neovitamin A then more should have been found in the caecum but this was not the case. This may have been due to a high rate of destruction in the caecum which could eliminate small differences in the total amount of vitamin reaching it. Unpublished work in this laboratory has shown that very rapid destruction of vitamin A occurs upon incubation with caecal contents.

The proportion of neovitamin A in the liver was rapidly reduced during depletion to a very low level, presumably by isomerization in the liver. This conversion of neovitamin A to all-*trans* appears to be efficient because there was no detectable difference in depletion rates whether the proportion of neovitamin A in the liver was initially high or low. The kidney does not seem able to perform this isomerization easily because the proportion of neovitamin A remained fairly high until the later stages of depletion.

Large oral doses of neovitamin A resulted in a higher proportion of this isomer in the liver stores than did smaller doses. The most likely explanation for this observation is that the intestine absorbs the same proportion of isomers, regardless of size of dose, but the large doses provide greater absolute amounts of neovitamin A to be isomerized by the liver; hence there is a delay before this is fully accomplished.

These experiments suggest that while *cis* isomers can be absorbed by the intestine they are converted to all-*trans* in the liver before they are utilized by the body. A similar conclusion was reached by Dowling and Wald (16) and Plack (14), both of whom worked with the 11-*cis* isomer. Plack was unable to decide from his results whether the low biological activity of the 11-*cis* isomer (23% of that of all-*trans*) was due to 23% efficient isomerization followed by fully efficient absorption, 23% efficient absorption followed by fully efficient conversion, or a combination of both factors. The results reported here suggest

that the lower potency of neovitamin A may best be explained by the latter alternative and indicate that the stomach and liver are the major sites of isomerization. The formation of neovitamin A from all-*trans* or vice versa also occurs in vitro. When either all-*trans* or neovitamin A is dispersed in water considerable isomerization occurs but at a much slower rate than in the above experiments (21).

The isomer composition of rat-liver oil is still a subject of controversy. Ames, Swanson, and Harris (22) found that rat-liver oil had a low relative biological potency. They attributed this to the presence in the oil of an equilibrium mixture of isomers, including those of low biological activity. On the other hand, Dowling and Wald (16) and Plack (14) found only all-*trans* and the isomer administered (11-*cis*) in the livers of rats. Biological assays in this laboratory (18) have not confirmed the results of Ames and his group. Furthermore the very low *cis* isomer content of vitamin A in rat liver reported here is not consistent with a low biological potency.

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OBSERVATIONS ON PROTEIN DIGESTION IN VIVO

II. DIETARY FACTORS AFFECTING THE RATE OF DISAPPEARANCE OF CASEIN FROM THE GASTROINTESTINAL TRACT[1]

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Abstract

The effects of various dietary changes on the emptying of solids and nitrogen from the stomach and on the disappearance of nitrogen from the gastrointestinal tract of rats trained to consume a single meal daily are described. When the animals were fed 5 g of food the presence of casein in the diet caused a general deceleration of stomach-emptying and altered the shape of the stomach-emptying curve. The presence of 50% of casein in the diet did not result in an accumulation of nitrogen in the intestine much above the amount found when a protein-free diet was fed. When dextrin was the dietary carbohydrate the diet emptied from the stomach as a homogeneous mixture, whereas when sucrose was the dietary carbohydrate the casein emptied from the stomach more slowly than did the other components of the diet. Raising the dietary level of fat to 50% caused a general deceleration of emptying and abolished the above-mentioned carbohydrate effect. No delay in stomach-emptying due to the presence of casein in the diet was noted when only 1.5 g of diet was fed. As the level of food intake was raised the total quantity of nitrogen emptying from the stomach per unit time increased although a greater percentage of the amount ingested emptied from the stomach when the level of food intake was low.

Introduction

Although the physiology of digestion and absorption has been extensively studied in higher vertebrates, particularly prior to the first World War, 1914–1918, the existing knowledge concerning in vivo digestion and absorption is still quite inadequate, a fact which was brought out recently by Fisher in his monograph on protein metabolism (1). Many of the experimental procedures used in studies of digestion have subjected the test animal to unnatural physiological conditions (2); consequently the results obtained may not be representative of normal gastrointestinal activity. Furthermore, in the earlier studies, the test diets were frequently incomplete and the components that were present were usually in a crude state (3), thus raising the question as to whether the results obtained were unduly influenced by the lack of certain dietary components or by the presence of contaminants in the ingested food.

In more recent investigations of these processes intact animals and purified dietary components have been used. Geiger (4), and Geiger, Courtney, and Geiger (5), studied the digestion of purified dietary proteins by intact rats and concluded that the rate of disappearance of casein from the gastrointestinal tract was greater than that of zein, but less than the rates for meat and fish proteins.

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Contribution from the Department of Biochemistry, University of Wisconsin, Madison 6, Wisconsin. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the National Live Stock and Meat Board, Chicago, Illinois, and Campbell Soup Company, Camden, New Jersey. Some of the crystalline vitamins were kindly provided by Merck Sharp and Dohme, Rahway, New Jersey.

In a study of the effects of certain carbohydrates on the gastrointestinal absorption of protein or carbohydrate in the rat, Dreisbach and Nasset (6) found that, 2 to 5 hours after the test meal had been fed, the quantity of protein recovered from the intestinal lumen was greater than the amount ingested. On the basis of these and other results (7) Nasset postulated (8) the existence of a homeostatic mechanism in which the gastrointestinal tract turns over a great deal of endogenous protein and that this "probably serves to even out temporary irregularities in the dietary supply of amino acids and to prevent gross changes in the amino acid pattern of the portal blood."

In contrast to these observations, Geiger, Humin, and Middleton (9) reported a low recovery of nitrogen from the gastrointestinal tract after feeding a test meal containing skim milk – protein to rats.

Gupta, Dakroury, and Harper (10) measured the rate of disappearance of a few proteins and an amino acid mixture from the gastrointestinal tract of the rat. Their procedure differed from that of Geiger *et al.* (4) in that a nutritionally complete diet was employed, 15% of which was the test protein (or amino acid mixture). The results of this study indicated that casein, beef protein, and an amino acid mixture disappeared from the gastrointestinal tract at similar rates. In the case of zein the rate of disappearance was found to be significantly less than that of the previously mentioned nitrogen sources. Their observations also indicated that the amount of nitrogen recovered from the gastrointestinal tract 4 to 5 hours after a test meal was fed to the rats was much less than the amount ingested.

The findings of the workers cited above, as well as those of other workers, revealed the need for a more systematic approach to the study of the over-all processes of digestion and absorption. The present investigation was therefore undertaken in order to provide necessary basic information which would serve as a foundation for further studies of this subject. The specific objective of the work reported in this paper was to determine the effect of various dietary factors on the rate of disappearance of a single dietary protein (casein) from the gastrointestinal tract. The factors studied were: the type of carbohydrate, the dietary levels of protein and fat, and the level of food intake.

Methods

Male albino rats weighing approximately 200 g were trained (in individual suspended cages with screen bottoms) to consume their total daily ration within a single 2-hour period. They were fed at the same time each day so as to provide maximum uniformity in the training pattern. Initially the rats lost weight, but at the end of 2 weeks on this regimen they had regained their starting weights, and were considered to be suitable for experiment (10).

The training diet consisted of: casein 15%, salts (11) 4%, corn oil 5%, water-soluble vitamin mixture (12) 0.25%, choline chloride 0.15%, and dextrin 75.6%. Fat-soluble vitamins A, D, and E were included in the corn oil to provide the following concentrations per 100 g of diet: A, 400 I.U.; D, 40 I.U.; and E, 10 mg.

The experimental diets described in Table I were lacking in water-soluble and fat-soluble vitamins since preliminary investigations had shown that the results of these short experiments were uninfluenced by the exclusion of these nutrients from the diets.

TABLE I

Composition of experimental diets

Diet	% casein	mg N/g	Type of carbohydrate	% fat
A	0	<1	Dextrin*	5 corn oil
B	0	<1	Sucrose	5 corn oil
C	15	21	Dextrin	5 corn oil
D	15	21	Sucrose	5 corn oil
E	30	42	Dextrin	5 corn oil
F	30	42	Sucrose	5 corn oil
G	50	70	Dextrin	5 corn oil
H	50	70	Sucrose	5 corn oil
I	15	20	Dextrin	25 lard
J	15	20	Sucrose	25 lard
K	15	20	Dextrin	50 lard
L	15	20	Sucrose	50 lard
M	0	<1	Dextrin	50 lard

*Autoclaved cornstarch.

Preliminary trials had also revealed that a well-trained rat would consume 5 g of food within 20 minutes. Therefore, on the day of the experiment, zero time was fixed at 20 minutes after the animals had been offered the test diet, and 5 g was chosen as the standard weight of diet to be fed in all experiments except those in which the effect of the level of food intake was studied. During the 20-minute feeding period spilled food, collected on paper towels placed under the cages, was returned to the food cups before the time limit had been reached.

Rats were then killed by chloroform anesthesia at zero time and at various intervals (five rats at each interval) thereafter, as designated in the results. The stomach and small intestine were excised and the contents of each were washed into separate evaporating dishes, each of which was lined with a weighed sheet of nitrogen-free plastic wrapping material.[2] After the samples had been dried and weighed, the nitrogen content of each entire sample was determined by the Kjeldahl method, using mercuric oxide as the catalyst.

The method of preparing the samples for analysis, as described above, differs from the procedure described by Gupta *et al.* (10), who did not analyze the entire sample (except in their study of zein) but diluted the total contents to known volume, homogenized, and analyzed aliquot portions of the fine suspension. Quite good replication was obtained using this procedure, but it was subsequently found that, owing to the relative insolubility of the casein which tended to precipitate out in the homogenate, the values for total nitrogen of the stomach contents were somewhat low. During the testing of diets C and D, pH measurements were made on the contents of the stomach and small intestine. This was accomplished by making incisions at the cardiac and

[2]"Saran-Wrap", Dow Chemical Company.

pyloric ends of the stomach and inserting the electrode of a single-electrode pH meter into these openings. The natural openings of the stomach were held closed by hemostats during this process. Rough pH measurements of duodenal, jejunal, and ileal contents were also taken by slitting the small intestine lengthwise and touching the electrode to its inner surface at various points.

In the early stages of the present investigation attempts were made to prevent the experimental animals from eating their feces. Tail cups, the use of which is described by Barnes *et al.* (13), were tried, but it was found that under such conditions gastric emptying was greatly delayed in a large number of rats and was completely halted for as long as 24 hours in a few. Immobilizing cages, as well as more fanciful devices, produced similar effects. Attempts to adapt adult animals to these devices were unsuccessful.

Later experiments indicated that the maximum quantity of fecal nitrogen eaten by any rat was too small to introduce a significant error into the determination, and as a result attempts to prevent coprophagy were discontinued.

Results

I. Effect of Protein

The effect of the level of dietary protein on the rate of stomach-emptying is most clearly demonstrated by the results obtained with diets containing dextrin as the carbohydrate (Fig. 1). With sucrose as the carbohydrate the results are complicated by the differential rates of emptying of carbohydrate and protein as will be shown later.

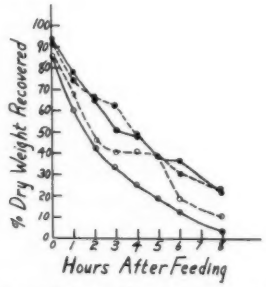


FIG. 1. The effect of dietary protein level on the rate of emptying of total solids from the stomach. The diets contain dextrin as the carbohydrate. Diet A ○——○ (protein-free), diet C ○ – – ○ (15% of casein), diet E ●——● (30% of casein), diet G ● – – ● (50% of casein).

The protein-free diet (A) emptied most rapidly from the stomach; the emptying curve is quite smooth and exponential in shape and the stomach appears to have been essentially empty 8 hours after the test diet was fed. In this experiment, and in all others in which a protein-free test meal was fed, there was no significant accumulation of nitrogen in the stomach at any of the time intervals tested. (The quantity of nitrogen recovered was always between 5 and 10 mg.)

In no case when a diet containing casein was fed was a smooth exponential curve for stomach-emptying obtained. Diet C (containing 15% of

casein) emptied from the stomach somewhat more slowly than diet A, the difference being more pronounced from the 3rd through the 5th hours after feeding. During this period stomach-emptying ceased entirely until after the 5th hour. Diet E (containing 30% of casein) passed from the stomach at a still slower rate than did diet C. Diet G (containing 50% of casein) emptied at essentially the same rate as diet E.

The effects of diets containing different levels of casein on the quantity of nitrogen in the intestinal contents at successive intervals after feeding are shown in Table II. Only the results obtained with diets containing dextrin are

TABLE II

Effect of variation in dietary protein level and fat level on the quantity of nitrogen in the small intestinal contents

Hours after feeding	mg N in intestinal contents				
	Diet A,* 1 mg N‡	Diet C,* 100 mg N‡	Diet E,* 200 mg N‡	Diet G,* 350 mg N‡	Diet K,† 100 mg N‡
0	8	9	12	17	10
1	7	12	12	14	9
2	8	13	12	10	9
3	11	15	11	11	9
4	10	9	11	9	11
5	11	8	7	11	10
6	9	7	8	10	14
8	9	8	8	9	13

*Contains 5% of fat.
†Contains 50% of fat.
‡Quantity of nitrogen in 5 g of diet.

shown because results obtained with sucrose as the carbohydrate were similar. The quantity of nitrogen in the intestinal contents remained consistently low both throughout the experimental period and irrespective of the percentage of casein in the diet. However, there was some tendency for more nitrogen to accumulate during the first 2 hours when the diet contained 50% of casein, although the difference was not statistically significant ($P > 0.05$). It was also observed that the total quantity of solids in the intestine remained relatively constant during these experiments, and did not rise significantly above values obtained with fasted animals ($P > 0.05$ at all intervals).

In these experiments the pH in the duodenum was approximately 6.8 while that in the jejunum and ileum lay between 7.0 and 8.0. These values remained constant in all experiments and at all time intervals tested.

Figure 2 provides a comparison of the total milligrams of nitrogen disappearing from the entire upper gastrointestinal tract (stomach and small intestine inclusive) at successive intervals after feeding diets containing different levels of casein. The quantity of nitrogen disappearing from the upper gastrointestinal tract within a given time interval was substantially greater when diet G (containing 50% of casein) was fed than when diet C (containing 15% casein) was fed. Although the curve for diet G appears to level off between the 7th and 8th hours after feeding, it actually continues its former steep ascent after the 8th hour. Only enough of the curve is shown to provide a comparison with

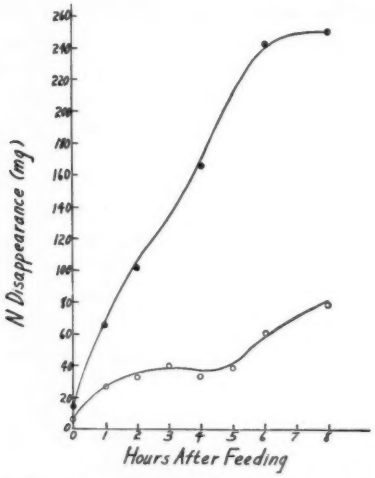


FIG. 2. The effect of dietary protein level on the rate of disappearance of nitrogen from the gastrointestinal tract between the cardiac sphincter and ileocecal junction. The diets contain dextrin as the carbohydrate. Diet C ○——○ (15% of casein), diet G ●——● (50% of casein).

the curve for diet C. The curve for diet E (containing 30% of casein) would fall midway between these two curves, so despite the fact that diets E and G emptied from the stomach at about the same rate on the basis of the percentage of ingested nitrogen emptying per unit of time (Fig. 1), the total amount of protein that emptied was much greater in the case of diet G.

II. Effect of Type of Carbohydrate

In Figs. 3–6 diets A, C, E, and G are compared with diets B, D, F, and H with regard to their rates of emptying from the stomach. The only difference between the two series of diets is that the latter contained sucrose instead of dextrin as the carbohydrate.

Figure 3 shows that protein-free diets A (dextrin as carbohydrate) and B (sucrose as carbohydrate) emptied from the stomach at closely similar rates;

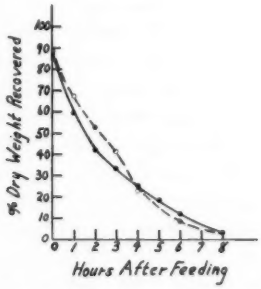


FIG. 3. The effect of type of carbohydrate on the rate of gastric emptying. Diet A ●——● (dextrin) (protein-free), diet B ○ – – ○ (sucrose) (protein-free).

at no time interval were the differences between them statistically significant ($P < 0.05$).

The curves in Figs. 4A and 4B show the rates of passage from the stomach of dietary nitrogen and total solids respectively. A comparison of the curves for diet C indicates that nitrogen and total solids emptied at the same rate when dextrin was the carbohydrate. When dextrin was replaced by sucrose, however (diet D Figs. 4A and 4B), the total solids emptied from the stomach

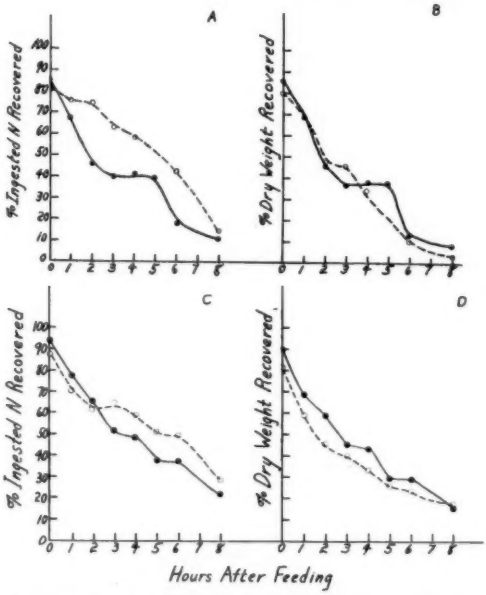


FIG. 4. The effect of type of carbohydrate on the rate of gastric emptying.
(A) and (B). Diet C ●——● (dextrin) (15% of casein), diet D ○ - - ○ (sucrose) (15% of casein).
(C) and (D). Diet E ●——● (dextrin) (30% of casein), diet F ○ - - ○ (sucrose) (30% of casein).

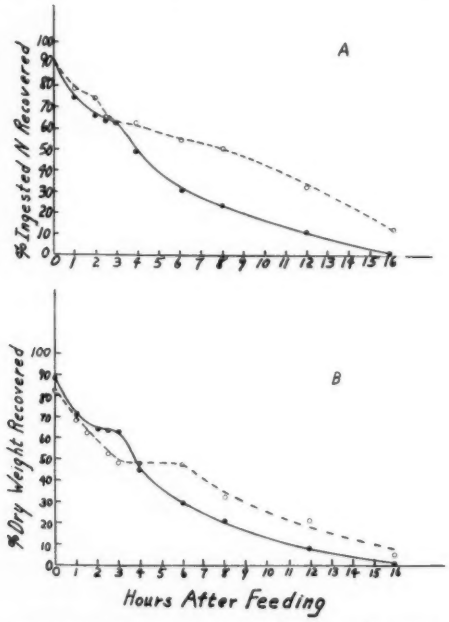
much more rapidly than did the nitrogen. The difference between these two curves is significant at all points from the 1st to the 8th hours after feeding ($P < 0.05$). In Fig. 4B the two curves are closely similar for the first 2 and last 2 hours of the experiment; but the delay in stomach-emptying during the intervening hours, which appears characteristic of diet C (containing dextrin), was relatively slight for diet D (containing sucrose).

Raising the dietary level of casein to 30% (diets E and F) produced the results shown in Figs. 4C and 4D. When diet E was fed, the dietary nitrogen and total solids passed from the stomach at similar rates (the percentage of nitrogen in the stomach contents, therefore, remaining constant) as had been previously noted with diet C in Figs. 4A and 4B. The rates of emptying of diets E and F (Fig. 4C) during the first 2 hours after feeding were not significantly different ($P > 0.05$). From the 3rd through 8th hours the nitrogen of

diet F (sucrose as carbohydrate) emptied from the stomach at a slower rate than did that of diet E (dextrin as carbohydrate). Although this difference is of doubtful statistical significance the consistency of its occurrence in repeated experiments lends it biological importance.

From Fig. 4D it appears that the total solids of diets E and F passed from the stomach at essentially the same rate, these results being similar to those shown in Fig. 4B for diets C and D. Figures 4C and 4D show that, as was previously observed with diet D (Figs. 4A and 4B), when diet F was fed the rate of disappearance of dietary nitrogen from the stomach was significantly slower than that of the total solids at all intervals between 0 and 8 hours after feeding ($P < 0.05$).

The effects of increasing the dietary level of casein to 50% (diets E and F) are shown in Figs. 5A and 5B. Comparison of the curves for diet E in Figs. 5A and 5B shows again that when dextrin was the carbohydrate the protein and



Figs. 5A and 5B. The effect of type of carbohydrate on the rate of gastric emptying. Diet G ●——● (dextrin) (50% of casein), diet H ○ – – ○ (sucrose) (50% of casein).

other components of the diet passed from the stomach at similar rates. Replacement of the dextrin in diet G with sucrose (diet H) again resulted in a more rapid exit from the stomach of the non-nitrogenous components of the diet.

There was no significant difference between the rates at which the nitrogen of diets G and H emptied from the stomach during the first 3 hours after feeding (Fig. 5A) ($P > 0.05$). However, between the 3rd and 16th hours

after feeding, the nitrogen of diet H emptied at a significantly slower rate than did that of diet G.

From Fig. 5B it can be seen that approximately 15% more of the total solids ingested had emptied from the stomach by the 3rd hour when sucrose was the carbohydrate than was the case when dextrin was the carbohydrate (diet G). This situation was reversed at the 6th hour, and thereafter the total solids of the diet containing dextrin emptied consistently more rapidly than did those of the diet containing sucrose, although the difference was of doubtful statistical significance.

The magnitude of the effect of carbohydrate on the emptying of protein is most clearly illustrated in Fig. 6. The curves in this figure represent the

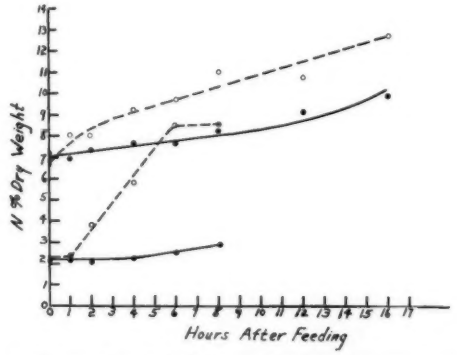


FIG. 6. The effect of type of carbohydrate on the nitrogen concentration of the gastric contents. The curves starting at 7% on the ordinate are for diets G and H, while the curves starting at 2% on the ordinate are for diets C and D.

$$\text{N\% dry weight} = \frac{\text{mg N}}{\text{mg dry wt.}} \times 100.$$

Sucrose as carbohydrate O- -O, dextrin as carbohydrate ●——●.

changes in the concentration of nitrogen in the stomach contents at intervals after feeding. Reference to the curves for diets which contained 15% of casein shows that when dextrin was the carbohydrate (diet C) the concentration of nitrogen in the stomach contents (N% dry weight) remained relatively constant throughout the experimental period. When sucrose was the carbohydrate (diet D), however, the nitrogen concentration had increased, by the 6th hour after feeding, until it was fourfold greater than that observed for the diet containing dextrin. This increase first became statistically significant at the 2-hour interval ($P < 0.05$).

The determination of pH as described previously yielded interesting results. When diet C, containing dextrin, was fed the pH of the contents of the antrum was 2.0 to 3.0 while that of the contents of the fundus was 5.0 to 6.0, and these values remained constant throughout the experiment. Of pertinence to these findings is the observation that the stomach contents were in the form of a discrete firm mass, the size of which decreased as digestion and gastric emptying progressed.

Replacement of dextrin by sucrose caused significant changes both in the pH and in the consistency of the stomach contents. The stomachs of rats that had eaten diet D appeared greatly distended with water and contained much less undissolved solid than was observed in the case of those that had eaten diet C. During the first 3 hours after feeding, a pH value of approximately 5 was obtained in all parts of the stomach of rats receiving diet D. A decrease to pH 4 was noted during the 4th hour, and by the end of the experiment, when only a small quantity of diet remained in the stomach, the pH had dropped to approximately 3.

III. Effect of Fat Level and Type of Carbohydrate

In a study of the combined effects of variations in the dietary level of fat and the type of carbohydrate on the passage of protein and total solids from the stomach, diets I and J (25% lard) and K and L (50% lard) were employed. Lard was substituted for corn oil in these experimental diets in order to avoid the use of a fat that was liquid and which, therefore, would tend to separate from the other components of the diet. In order to obtain a basis for comparison between diets containing lard and those containing corn oil an experiment was performed using a diet containing 5% of lard with dextrin as the carbohydrate. The resulting curves for the emptying of dietary nitrogen and total solids from the stomach were found to be superimposable on the curves for diet C in Figs. 2A and 2B, and for this reason are not presented. From these findings it was concluded that 5% of corn oil and 5% of lard had similar effects on stomach-emptying.

The curves for diet I (dextrin as carbohydrate) in Figs. 7A and 7B show that this diet emptied from the stomach at a substantially slower rate than did diet C which contained only 5% of fat (Figs. 3A and 3B). As was noted with diet C the total solids and dietary nitrogen passed from the stomach at closely similar rates. With sucrose as the carbohydrate (diet J) the emptying pattern was similar to that of diet D (Figs. 3A and 3B), i.e., the total solids in the stomach emptied at a more rapid rate than did the dietary nitrogen.

Figure 7A shows that the nitrogen of diets I and J emptied at closely similar rates for the first 3 hours after feeding, there being no significant difference between the curves during this period ($P > 0.05$). From the 3rd through 5th hours after feeding, the nitrogen of diet I emptied more rapidly than did that of diet J, the difference being statistically significant at the 3- and 4-hour intervals ($P < 0.05$). However, the total solids of diet J emptied at a consistently more rapid rate than was the case for diet I although the patterns of emptying were quite similar (Fig. 7B).

The effect on stomach-emptying of raising the dietary fat level to 50% (diets K, L, and M) is shown in Figs. 7C and 7D and Fig. 8. From Figs. 7C and 7D it can be seen that there was no significant difference between the rates of emptying of diets K (dextrin as carbodydrate) and L (sucrose as carbohydrate). The delay in emptying observed between the 2nd and 4th hours after feeding these diets is similar to that obtained with diet C (5% fat with dextrin, Figs. 3A and 3B), but there was a general decrease in the rate of emptying of the high-fat diets (Figs. 7C and 7D) compared with that of diet C.

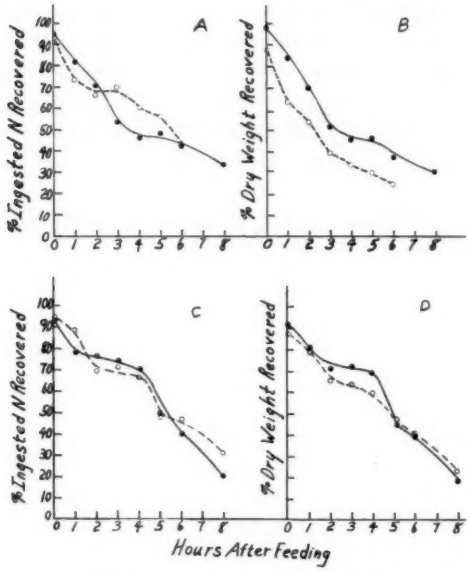


FIG. 7. The effect of dietary level of fat on the rate of gastric emptying.
(A) and (B). Diet I ●——● (dextrin) (15% casein, 25% fat), diet J ○ – – ○ (sucrose) (15% casein, 25% fat).
(C) and (D). Diet K ●——● (dextrin) (15% casein, 50% fat), diet L ○ – – ○ (sucrose) (15% casein, 50% fat).

FIG. 8. The effect of dietary level of fat on the rate of gastric emptying. Diet M ●——● (dextrin) (protein-free, 50% fat).

Comparison of Fig. 8 with the curve for diet A in Fig. 3 reveals that protein-free diet M, which contained 50% of fat, emptied from the stomach at a significantly slower rate than did protein-free diet A, which contained 5% of fat. It is also notable that the emptying patterns of diets A and M failed to show a prolonged period of complete cessation of stomach-emptying, a phenomenon which was observed with high- and low-fat diets containing 15% of casein (diets K and C). However, a short period of cessation did occur between 1 and 2 hours after feeding the high-fat protein-free diet.

Comparison of the intestinal results for diets K and C (Table II) shows that although diet K contained 45% more of fat than did diet C the quantity of nitrogen in the small intestinal contents remained at the same low level for both diets throughout the experimental period. This low level corresponds to that observed for diet C, which contained no protein and 5% of fat.

IV. Effect of Level of Food Intake

The rates of emptying from the stomach of various quantities of diet C are compared in Fig. 9. When 1.5 g of diet C were fed, gastric emptying proceeded

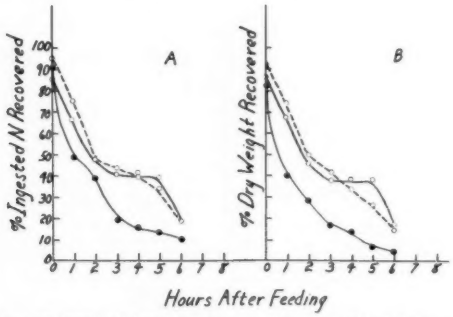


FIG. 9. The effect of level of food intake on the rate of gastric emptying of diet C. 1.5 g fed ●——●, 3.0 g fed ○ – – ○, 5.0 g fed ○——○

more rapidly than when 3.0 or 5.0 g were fed. The over-all rates of emptying at the latter two levels of intake were closely similar, although the delay in emptying, occurring between the 3rd and 5th hours after feeding 5.0 g of diet was less pronounced when 3.0 g were fed. This period of delay was totally absent when the level of intake was 1.5 g. Expressing these results as milligrams of nitrogen emptied rather than as percentage of ingested nitrogen discloses that the quantity of nitrogen passing from the stomach per unit of time increased with increasing level of food intake.

Discussion

I. Effect of Protein

From the curves presented in Fig. 1 it is evident that the inclusion of protein in the diet had a pronounced effect on the rate of passage of food from the stomach. The presence of protein caused the emptying process to be intermittent, with periods of deceleration (and in some cases of complete cessation) of emptying occurring at various intervals during the period after feeding. Maziyevskii and London (14) noted that periodic cessation of stomach-emptying occurred in dogs which had been given a test meal of starch. In the present investigation a similar phenomenon was found to be brought about by dietary protein. In addition, the added protein caused an over-all deceleration in the rate of emptying of the test diet from the stomach. This effect was increased somewhat when the level of protein in the diet was raised to 30%.

Among the many factors which must be considered in interpreting these effects are: (*a*) the alteration in the physical consistency of the diet due to

the increased casein content; (*b*) the increasing quantities of amino acids absorbed per unit of time with higher protein intakes; (*c*) the fact that higher levels of dietary protein in the stomach contents may have more completely buffered the hydrochloric acid secreted by the parietal cells, thus keeping the pH well above that required for the maximum digestive activity of pepsin. These three factors may in combination exert an inhibitory effect on stomach-emptying which varies with the level of protein in the diet. The lack of significant differences between the rates of emptying of diets containing 30% and 50% of casein suggests that the maximal inhibitory effect of protein on stomach-emptying is reached when the diet contains about 30% of casein.

The decelerating effect of added dietary protein on gastric emptying was also noted by Annegers and Ivy (15), who observed that a level of 55% of protein in the diet caused an over-all delay in stomach-emptying. These results, as well as those of the present investigation differ from those of Rosenthal and Nasset (7), who found that the presence or absence of protein in the diet did not significantly affect stomach-emptying. Their failure to observe a protein effect may be attributable to the fact that they fed only 1.5 g of diet as compared with the 5.0 g used in the present study. As will be mentioned later, the effect of the level of food intake on stomach-emptying is a variable which must be considered in studies of dietary effects on gastrointestinal processes.

The results shown in Table II reveal that when casein was the dietary protein, nitrogen did not accumulate in the intestinal lumen under any of the conditions of the present investigation but was absorbed extremely rapidly. The significance of this finding becomes apparent from examination of Fig. 2 which shows that the gastrointestinal tract was able to digest and absorb far larger quantities of dietary nitrogen (per unit of time after feeding) when the diet contained 50% of casein than when it contained 15% of casein. Thus the absorptive process was not the limiting factor in determining the rate of disappearance of dietary nitrogen from the gastrointestinal tract when the source of this nitrogen was casein. That the disappearance of the dietary casein from the small intestine occurs by means of absorption, and not by passage into the large bowel, is indicated by the observations of Mitchell and Bert (16) and Crampton and Rutherford (17), who reported a negligible increase in fecal nitrogen with a large increase in casein intake. It was postulated that even this slight increase may have been due to an increase in bacterial protein. The intestinal data of the present investigation are in agreement with the results of Gupta *et al.* (10), and with those of Rosenthal and Nasset (7). The conclusion of Rosenthal and Nasset (7) regarding the significance of the contribution of protein by the intestine to the chyme is, however, open to question. In the present experiments the capacity of the intestine to cope with a range of 0 to 350 mg of dietary nitrogen was tested, while Rosenthal and Nasset (7) tested only over a range of from 1 to 55 mg of nitrogen. Therefore their statement that "the lower the nitrogen intake the greater the percentage recovery" has little significance except in its support of the conclusions reached in the present investigation. The term "percentage" was not used in Table II because the

quantity of endogenous nitrogen constantly present in the intestine is equivalent to a much higher percentage of the dietary nitrogen when the protein intake is low.

Nasset's statement that diets containing protein cause greater stimulation of digestive secretions than do protein-free diets (18) is not supported by the results of the present investigation. Table II shows, as stated previously, that the feeding of diet C (15% of casein) did not result in a significantly greater accumulation of nitrogen in the intestinal contents than was the case when diet A (protein-free) was fed. Therefore, if intestinal secretions were stimulated to any great extent by dietary protein under the conditions of the present investigation, the secreted material must have been rapidly absorbed. Also, as shown in Fig. 6, the percentage of nitrogen in the stomach contents did not increase significantly (over the percentage originally present in the diet) with time after feeding diet C. This indicates that gastric secretions were not stimulated specifically by the dietary casein.

Admittedly, the foregoing conclusions are based on indirect evidence and more accurate information about this problem is required. More conclusive information concerning the contribution and turnover of the gastrointestinal secretions can be obtained using isotopic tracer techniques. Such an investigation is currently being considered.

II. The Combined Effects of Protein and Carbohydrate

In evaluating the results shown in Fig. 3 through Fig. 6 the physical differences between the two carbohydrates being tested should be kept in mind, i.e. that sucrose has a much lower molecular weight, and is much more water-soluble than dextrin. These physical differences did not have any noticeable effect on the rates of emptying of protein-free diets A and B from the stomach; however, when casein was present in the diet the rates of passage from the stomach of dietary nitrogen and total solids were influenced by the type of dietary carbodydrate. This is most clearly illustrated by Figs. 4A and 4B and Fig. 6, which serve as the basis for the following explanation of this phenomenon.

When a diet containing dextrin and casein was ingested these components formed a firm mass in the stomach and remained intimately mixed in the same proportion in which they were present in the diet. This is shown by the fact that the percentage of nitrogen in the dry stomach contents (Fig. 6) from rats fed diet C remained quite constant throughout the experimental period. Although the digestion of the protein in this mass must have proceeded slowly in the cardiac portion of the stomach owing to the relatively high pH there, normal gastric digestion could occur in the antrum where the pH was between 2 and 3, near that required for the optimum activity of pepsin. As the contents of the antrum were partially digested they apparently passed through the pyloris into the duodenum; meanwhile more food moved into the antrum where the process was repeated.

When diet D, containing sucrose as the carbohydrate, was fed, a separation of the diet into two phases occurred in the stomach. The sucrose dissolved in the water which was drawn into the stomach presumably because of the high

osmotic pressure exerted by this diet (19), whereas the casein failed to go into solution. Owing to the resulting high fluid content of the stomach contents there was no difference between the pH in the antrum and that in the fundus. The pH of the antrum was as high as that of the fundus for the first few hours after feeding. This would cause precipitation of the casein and would probably cause some inhibition of the gastric digestion of casein by pepsin. The fluid portion of the contents, however, was apparently able to pass through the pyloris into the intestine, leaving much of the casein behind. This is evident from the fact that the curves for the percentage of nitrogen in the stomach contents of rats that were fed diets containing sucrose rise sharply (Fig. 6). The high concentration of sucrose in the fluid passing into the intestine might also have caused a reduced rate of gastric emptying (20).

The above explanation appears to hold also for the results shown in Figs. 4C and 4D for diets E and F as well as those shown in Figs. 5A and 5B for diets G and H, although the previously noted effects were less pronounced with these diets presumably due to their higher casein content. Since the casein was added in place of carbohydrate, the contribution of carbodydrate to the over-all effect was undoubtedly diminished. The altered consistency of the diet caused by the added protein may also have been a contributory factor.

Rosenthal and Nasset (7) also observed effects of the type of dietary carbohydrate on stomach-emptying. They found that a diet containing dextrin emptied from the stomach more rapidly than did a diet containing fresh banana as the carbohydrate source. In earlier experiments Gupta *et al.* (10), using a diet similar to diet D, obtained a curve for the rate of emptying of dietary nitrogen from the stomach which fell more sharply than that obtained in the present study. The more rapid fall they observed can be accounted for partly by the sampling procedure they employed, which resulted in somewhat lower nitrogen recovery (see procedure), partly by the fact that the food intake of their rats was not constant and was somewhat less than 5 g, and partly by the fact that they studied fewer time intervals after feeding of the test meal, a procedure that tends to obscure irregularities in the time curve.

III. Effect of Fat Level

It is evident from Figs. 7A and 7B that the increase in dietary fat level from 5 to 25% caused a general deceleration in gastric emptying. However, the effects previously noted as being due to the differences in type of carbohydrate are still quite apparent and may be explained in the same manner as were the results shown in Figs. 4A and 4B; i.e., diet I remained as a homogeneous mixture throughout its digestion and passage from the stomach while the sucrose of diet J dissolved, and passed from the stomach at a more rapid rate than did the dietary casein. If pictured, the curves for the percentage of nitrogen in the dry stomach contents for diets I and J would follow a pattern similar to those for diets C and D in Fig. 7.

The inhibitory effect of a high level of dietary fat on stomach-emptying is clearly illustrated in Figs. 7C and 7D as well as in Fig. 8. The findings of Farrell and Ivy (21) suggest that this effect may be due to the action of

enterogastrone. No effect of the type of carbohydrate on stomach-emptying was evident when the diet contained 50% of fat. A combination of three factors may have been responsible for the lack of carbohydrate effect with this level of fat. These are: a reduction in the physical differences between the diets due to the addition of fat (the consistencies of diets K and L were closely similar), a decrease in the carbohydrate content of the test diets, and the stronger influence on stomach-emptying of a fat-activated inhibitory mechanism. The rather substantial difference between the shape of the curve for diet K in Fig. 7D and that for diet M in Fig. 8 indicates that the effect of the presence of 15% of casein (added in place of dextrin) in diet K was not masked by the high level of dietary fat. London and Sivre (22) reported that fat emptied from the stomach of dogs at a slower rate during the first 2 or 3 hours after feeding than during the remaining period of digestion. Thus their data apparently resemble the results shown in Figs. 7C and 7D, although it is believed that the stomach-emptying pattern observed in the latter case was the result of the combined effect of protein and fat and was not due to the effect of fat alone.

As indicated by the results for diet K in Table II the presence of a high concentration of fat in the diet did not appear to influence either the speed of absorption of gastric digestion products or the quantity of intestinal secretion.

IV. Effect of Level of Food Intake

The results shown in Figs. 9 and 10 indicate that the level of food intake has a definite effect on the rate of emptying of food from the stomach. When the rate of emptying was expressed in the manner shown in Fig. 9, i.e., when percentage recovery is plotted versus time after feeding it is evident that a very low level of food intake (1.5 g) resulted in a more rapid rate of emptying. Also, the effects of the presence of dietary protein on stomach-emptying are not evident with this level of food intake; in fact, the curve for the percentage recovery of ingested dry weight at the 1.5 g level of intake (Fig. 9B) is quite similar in shape to the curve for diet A (protein-free) in Fig. 3. The over-all rate decreased as the food intake was increased up to 3.0 g, and the shape of the curve altered somewhat, with a fairly sharp decrease in the negative slope occurring at the 2-hour interval. This indicates that the quantity of food eaten in this case was such that the presence of protein in the diet had a noticeable effect on stomach-emptying. Raising the level of intake to 5.0 g caused no further change in the over-all rate of emptying. However, the effect of the presence of dietary protein on stomach-emptying becomes clearly evident at this level of intake. Therefore, the conclusion of Rosenthal and Nasset (7) that the presence or absence of protein in the diet has no effect on stomach-emptying is open to question. Although a greater percentage of ingested food emptied from the stomach per unit of time when 1.5 g of diet were fed than when 3.0 or 5.0 g were fed, the total quantity of food emptied from the stomach per unit time increased as the level of food intake increased (Fig. 10). This indicates that the stomach responds to the increased load associated with a

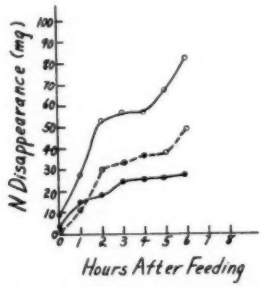


FIG. 10. The effect of level of food intake on the rate of gastric emptying of diet C. 1.5 g fed ●——●, 3.0 g fed ○ - - ○, 5.0 g fed ○——○.

higher level of food intake and that this factor can influence greatly the results of digestion studies of the type outlined in this paper.

Taken all together the results of these experiments indicate clearly that both the type and the quantity of diet consumed influence the rate at which the ingested substances pass into the body fluids.

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A GENERAL CHROMATOGRAPHIC SURVEY OF AROMATIC COMPOUNDS OBTAINED FROM URINE[1]

D. M. SMITH,[2] R. M. PAUL, E. G. MCGEER, AND P. L. MCGEER

Abstract

Aromatic compounds contained in both normal and schizophrenic urines have been extracted by several methods and the resultant extracts examined by two-dimensional paper chromatography with a variety of spray reagents. A comprehensive map plus tables summarize the data obtained on over 200 "aromatic" spots found in various urine extracts. Data are given on fractionation of the extracts. To allow comparison of one extract with another, an attempt was made to maintain quantitative control of the extraction, fractionation, and chromatographic procedures.

Introduction

The field of aromatic metabolism has long been of unusual interest because disturbances occur in the normal metabolic paths in a wide variety of pathological conditions, including deficiency diseases such as ascorbic acid and pyridoxine deficiency, metabolic diseases such as phenylketonuria and alkaptonuria, and organ failures such as liver and kidney disease. Schizophrenia is another disease where investigation of aromatic metabolism is of current interest. Buscaino (1), Holmes (2), Hoffer (3), and others have suggested a psychotoxic etiology for schizophrenia based on the analogy between the mental effects produced by known aromatic hallucinogens and the symptoms of schizophrenia. Further study has shown the difficulty of proving or disproving these hypotheses by revealing the very large number of aromatic compounds in body fluids. A major part of the efforts of our laboratory is directed towards the development of satisfactory methods for the extraction of aromatic compounds in urine, for the fractionation of such extracts, and for the chromatographic characterization and identification of the individual compounds. This paper extends previous reports from this laboratory (4, 5, 8) by emphasizing the quantitative aspects of the extraction and chromatographic procedures and by providing chromatographic characterization of about 125 spots, in addition to the 95 previously reported. These additional spots have been revealed through improvements in chromatographic technique.

Methods and Procedures

A. General

In this study, overnight urine samples from various groups of normals, schizophrenics, and phenylketonurics were used. In most cases samples from a number of individuals were pooled in an attempt to minimize the effects of

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diagnostic error, dietetic idiosyncrasies, and biological individuality. Unprocessed urine was unsatisfactory for detailed chromatographic work on aromatic compounds because of interference by salts, aliphatic urinary constituents, and other "contaminants". It was therefore necessary to extract the aromatic materials. The two basic methods employed to accomplish this were solvent extraction and adsorption on carbon followed by elution. In order to compare the contents of one extract with another, and with the fractions of the various extracts, it was desirable to have an accurate quantitative estimate of how the various aromatic compounds distributed themselves. We found no method which was completely suitable for this purpose, but we used three semiquantitative methods at different stages to estimate recoveries,

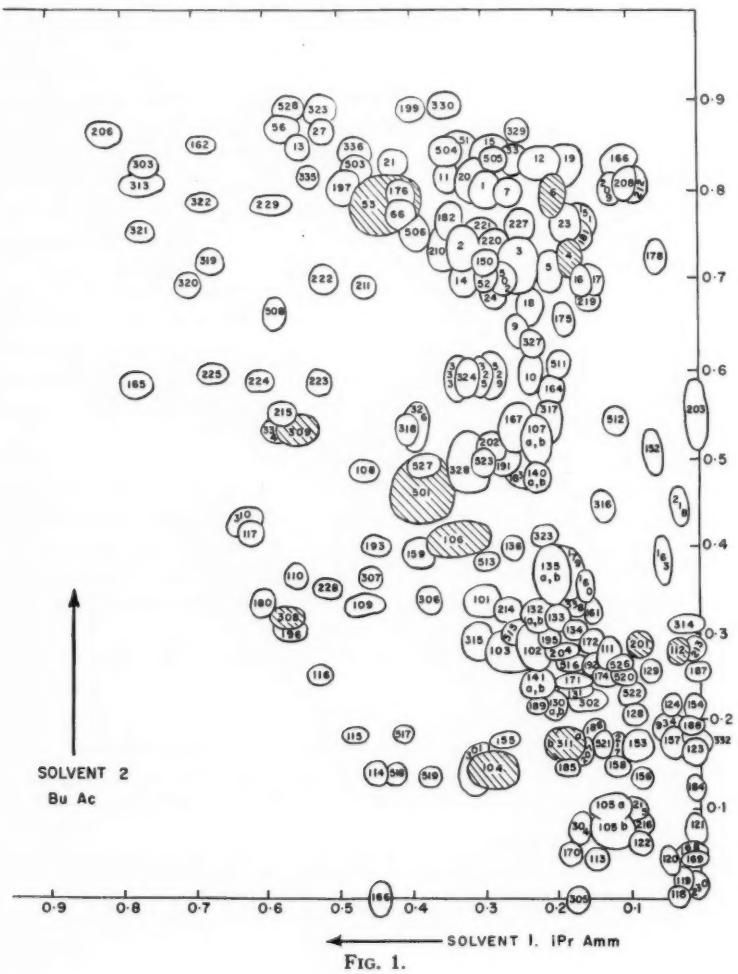

FIG. 1.

each with separate indications. These were free and total phenol content as determined by the Folin–Denis procedure, organic solids content, and direct ultraviolet spectral absorbance of a given volume of urine or extract.

As can be seen from the chromatographic map (Fig. 1) the proximity of compounds is such that fractionation of extracts was necessary to obtain high resolution on the chromatograms. Fractionation was achieved either by solvent separation or by column fractionation using Dowex-1 or Dowex-50W resins. Chromatograms were run in most cases in butanol/acetic acid/water (4:1:1) and isopropanol/ammonia/water (8:1:1). The chromatograms were all examined under ultraviolet light and then sprayed with one of nine spray reagents.

B. Methods of Quantitative Control

The obvious way to assess recovery of aromatics from urine is to assess the concentration of urine initially either by creatinine or volume output and relate the volume of extract obtained to the initial urine standard. Unfortunately, the variation in recovery of aromatics from carbon despite the use of apparently identical procedures made this method of limited value. The factors responsible for this variation are not understood by us, although we tried to evaluate the variation by measuring the free and total "phenols" in the initial urine and in the extract by the Folin–Denis procedure (6). Once the extract was obtained, fractionation was followed both by measurement of free and total "phenols" and by measurement of organic solids content. The latter was determined in the following way: 0.5 ml of sample in a weighing crucible was evaporated to constant weight using a vacuum oven at 50° C; the sample was then ashed in a muffle furnace at 600° C overnight, cooled in a desiccator, weighed, and the process repeated to constant weight. The content of inorganic and organic solids could then be calculated. Where only very small samples were available, as for example with column fractions, direct ultraviolet absorbance at 275 mμ was used as a quantitative measure. Differences in pH of the sample were found to have little effect on the absorbance.

Trial of both the Folin–Denis and ultraviolet absorbance methods on varying amounts of individual urines and extracts indicated that Beer's law was obeyed within reasonable concentration limits. Both methods suffer, however, from the failing that the various aromatic components in urines and extracts do not contribute equally to the result. Data obtained by the Folin–Denis method are cited as "phenols" and those obtained by the direct spectral method are called "aromatic content". Organic solids, though obviously a much more reliable criterion than either of the above methods, cannot be used for the important initial step of extracting aromatic compounds from urine.

C. Extraction Procedures

1. Adsorption on Carbon and Elution

Initially, crude urine was treated with charcoal which was then eluted with phenol-saturated water as recommended by Dalgliesh (8, 9). Trials of various amounts of carbon (Baker and Adamson, decolorizing carbon, code No. 1551)

per 100 ml of urine indicated that 5 g was usually sufficient to decolorize the urine completely. Trial at various pH's indicated that adsorption was excellent at pH 4 or less but was incomplete at higher pH's. Analyses of seven filtrates resulting from such treatment indicated that 5–12% of the free and 6–14% of the total "phenols" in the initial urine were not adsorbed. Chromatograms of one such filtrate, though difficult to interpret because of distortion by salt, indicated that histamine and histidine were present; this corroborates the report of Asatoor and Dalgliesh (9) that these imidazoles adsorb poorly on carbon. Use of excessive amounts of carbon led to difficulties in elution (Table I(*a*)) and use of smaller amounts led to much greater losses in the filtrate. The color of the filtrate is a reasonably good indicator of the completeness of adsorption as judged by "phenol content".

TABLE I

(*a*) Effect of amount of carbon on ease of elution*

Amount urine, ml	Grams carbon/ 100 ml urine	Recovery in three elutions	
		Free "phenols", %	Total "phenols", %
900	8	26†	21†
800	5	65	56

(*b*) Effect of carbon deactivation on adsorption–elution process‡

	Stearic-acid-deactivated carbon	Untreated carbon
Amount C used	50 g	50 g
% free phenols in filtrate	21%	8%
Amount additional C used	14 g	—
% free phenols in filtrate	12%	—
Recovery on three elutions of:		
Free phenols	40%	45%
Total phenols	36%	42%

*NOTE: Extracts prepared from aliquots of the same schizophrenic urine pool analyzing for 0.35 mg/ml free and 0.52 mg/ml total "phenols" on urine adjusted to specific gravity 1.020.
†Increased only to 47% and 42%, respectively, by threefold repeated re-elution.
‡NOTE: Each extract was made with 840 ml of a schizophrenic urine pool analyzing for 0.45 mg/ml free and 0.65 mg/ml total "phenols" on urine adjusted to specific gravity 1.020.

Since Asatoor and Dalgliesh (9) found with pure aromatic materials that deactivation of the carbon with stearic acid resulted in better recoveries, we compared deactivated carbon with untreated carbon, using equal aliquots of the same urine specimen (Table I(*b*)). The data indicate that the adsorptive capacity of carbon is decreased to 85% by deactivation. If sufficient carbon of each type is used to obtain nearly quantitative adsorption, the recovery is as good in an equal number of elutions from the untreated as from the deactivated carbon. Hence, untreated carbon was used for our work. The adsorption–elution process depends on the surface properties of the carbon which may vary from carbon to carbon made by different manufacturers and even, perhaps, from lot to lot. Our carbon was a Baker and Adamson product while Asatoor and Dalgliesh used a B.D.H. product.

The usual procedure in the preparation of extracts was as follows: The urine was acidified with hydrochloric acid immediately after collection. It

was filtered and a small sample reserved for analytical work. The urine was mixed with 5 g of carbon per 100 ml and the mixture was shaken and allowed to stand in a refrigerator overnight. The carbon was then shaken with phenol-saturated water (5–10 ml/g of carbon) for at least 1 hour. The elution could be speeded somewhat by heating to 40–50° C but the recoveries were not appreciably improved. The carbon was filtered, washed with water, and re-eluted.

The number of elutions varied from extract to extract. Two elutions resulted in a very poor recovery (40–45%). Four elutions were often sufficient to achieve 70–90% recovery of "phenols". The usual procedure was to elute four times, and re-elute if the initial recoveries were less than 60% of the free "phenols". All elutions and washings were combined and evaporated under vacuum at 50° C. Water was added and the concentration repeated. This process was continued until the distillate was phenol-free according to a ferric chloride test. Quantitative data on the recoveries in 29 extracts made by this general procedure are given in Table II.

TABLE II

Some extracts prepared by a standard carbon–phenol procedure

Extract No.	Type urine	Urine volume, ml	"Phenols"/ml*		Recovery of "phenols"		No. of elutions	Ratio: total/free "phenols" in:	
			Free	Total	Free, %	Total, %		Urine	Extract
5	Schizophrenic	3700	0.42	0.63	60	55	5	1.50	1.33
18	"	750	0.35	0.50	65	67	4	1.30	1.31
20	"	1400	0.46	0.58	62	60	5	1.26	1.22
26	"	180	0.38	0.57	57	55	6	1.50	1.45
27	"	150	0.37	0.55	55	55	6	1.48	1.48
37	"	800	0.37	0.54	65	56	4	1.46	1.28
46*a*	"	1750	0.26	0.38	75	65	4	1.50	1.30
46*b*	"	1700	0.28	0.42	70	62	4	1.50	1.34
46*c*	"	2000	0.33	0.50	88	80	4	1.53	1.39
47	"	1500	0.32	0.55	66	65	4	1.72	1.69
47*a*	"	500	0.32	0.55	67	72	4	1.72	1.87
48	"	1700	0.27	0.41	63	63	5	1.50	1.50
49	"	2000	0.39	0.59	88	80	4	1.51	1.39
56	"	45	0.39	0.59	65	59	4	1.51	1.37
Means			0.35	0.53	68	64		1.48	1.42
Means adjusted for urine volumes			0.35	0.51	70	65		1.50	1.39
4	Normal	1500	0.29	0.41	71	55	4	1.46	1.13
12	"	1000	0.30	0.45	72	75	4	1.50	1.54
13	"	1000	0.26	0.39	75	72	4	1.50	1.49
14	"	1000	0.31	0.47	60	60	4	1.52	1.50
40*a*	"	2450	0.30	0.49	75	75	4	1.63	1.63
40*b*	"	8000	0.25	0.35	79	73	4	1.39	1.29
40*c*	"	3100	0.27	0.44	86	68	4	1.63	1.29
40*d*	"	5500	0.18	0.30	88	71	4	1.67	1.32
41*a*	"	3450	0.26	0.42	92	75	4	1.61	1.39
41*b*	"	1700	0.38	0.58	77	68	4	1.52	1.31
41*c*	"	2000	0.25	0.34	67	77	4	1.36	1.61
41*d*	"	4000	0.38	0.47	82	70	4	1.57	1.34
41*e*	"	4200	0.18	0.25	82	89	4	1.39	1.51
Means			0.27	0.41	77	71		1.52	1.41
Means adjusted for urine volumes			0.25	0.38	81	73		1.52	1.37
28 Treated	Phenyl-	170	0.25	0.34	78	90	4	1.36	1.57
29 Untreated	ketonurics	1500	0.41	0.63	73	70	4	1.53	1.46

*For urines adjusted to specific gravity of 1.020.

There are three sources of loss of "phenols" in this extraction procedure. These are: (1) failure to adsorb on carbon. This was determined to be less than 14% if sufficient carbon were used; (2) failure of elution from the carbon; and (3) loss of volatile materials with phenol during the washing and concen-

tration of the eluant. One attempt was made to estimate how serious this third loss might be: 1500 ml of schizophrenic urine was acidified and concentrated at 50° C under vacuum to approximately 300 ml; the distillate was collected in a flask containing a base which was subsequently acidified and extracted with ethyl acetate. Analysis indicated loss of some 7% of the "free phenols" in the initial urine by this process which was similar to that used for driving phenol off the eluant. About 4.5% of these "free phenols" were recovered in the ethyl acetate extract.

2. Variations in the Carbon Adsorption–Elution Procedure

(*a*) *Adsorbent*

The carbon can be admixed with "Celite Hi Flo" diatomaceous earth filter aid to facilitate easy filtration; the "Celite" has little or no effect on the adsorptive capacity.

(*b*) *Eluting Solvent*

Kemali (10), Decker (11), and others have used ammoniacal organic solvent mixtures for elution from carbon. The effectiveness of Kemali's eluting mixture of water/butanol/ammonia/methanol (75:10:10:5) was compared with that of phenol-saturated water on separate aliquots of the same urine specimen. Use of 1.2 g of carbon per 100 ml of urine, as recommended by Kemali (10), led to adsorption of only about 30% of the "phenols" in urine and so 5 g per 100 ml of urine was used as in the extracts reported in Table II. Using elution conditions comparable to those used with phenol-saturated water, recoveries of less than 50% were obtained (see Table III). Recoveries of 65 to 75% could

TABLE III

(*a*) Recoveries with various eluants*

Eluting solvent	Recoveries of "phenols"		Comparable standard extract recoveries of "phenols"		
	Free, %	Total, %	No.	Free, %	Total, %
Kemali ($MeOH/BuOH/H_2O/NH_4OH$)	45	38.5	47, 47*a*†	66–67	65–72
Kemali ($MeOH/BuOH/H_2O/NH_4OH$)	45	45.5	56†	65	59
Methyl acetate/NH_4OH (7: 3)	48	47	37†	65	56

(*b*) Recoveries with phenol-saturated water and with various alkaline eluting solvents in column work

Eluting solvent	Recovery based on absorbance measurements, %
7% phenol in water	69
7% phenol in water	87
Kemali ($MeOH/BuOH/H_2O/NH_4OH$)	64
2% pyridine	61

*Standard conditions were used.
†For additional data on these extracts, see Table II.

be obtained by using very large volumes of the ammoniacal eluting mixture. Since such large volumes are difficult to handle, our preference remains with phenol-saturated water, even though steam distillation of the phenol is very

time-consuming. Analysis by Nessler's reagent (7) indicated that the extracts obtained by evaporation of Kemali-type eluants typically contained 0.8–2 mg of ammonia per 100 mg of organic solids.

Use of methyl acetate/aqueous ammonia (7:3) as the eluant gave a recovery of less than 50% (Table III(*a*)).

Two per cent pyridine in water, as recommended by Riegert (12) for the selective elution of imidazoles, also gave poor recoveries if reasonable volumes of eluant were used (Table III(*b*)). Chromatography indicated that there was no significant degree of fractionation in these extracts.

Better recoveries (65–75%) were obtained with smaller volumes of phenol-saturated water neutralized with sodium hydroxide than with the slightly acid phenol-saturated water. However, extracts obtained by this procedure were seriously contaminated with inorganic salts (10–15% of the total solids). Basic eluting media suffered from the disadvantage that colloidal carbon tended to come through into the filtrate. If the filtrates were concentrated under vacuum and an equal volume of ethanol added, the carbon coagulated and could be removed by filtration on paper which had been previously washed with ethanol.

Simple neutral organic solvents and various aqueous solutions of phenol containing less than 7% phenol were tried with the objective of obtaining fractionation. In most of this work, which was done on columns, recoveries were poor.

A basic eluting solvent is capable of eluting some additional material from carbon which has already been eluted repeatedly with acidic phenol-saturated water (cf. Table IV). The compounds so obtained are predominantly polar materials (low R_f in butanol/acetic acid/water (4:1:1)). Our practice at present is to elute the carbon three or four times with phenol-saturated water, wash it completely free of phenol and then elute it two or three times with Kemali's mixture or with 2% pyridine in water. The 2% pyridine has the advantage that traces of pyridine remaining after evaporation can be removed by ether or ethyl acetate extraction.

TABLE IV

Additional recoveries obtained using a basic eluant after phenol-saturated water

Recoveries of "phenols" with phenol water			Additional recoveries of "phenols" with basic eluant	
Free, %	Total, %	Basic eluting material	Free, %	Total, %
77	72	Phenol–water made to pH 7.5–8 with NaOH	13	14
82	77		14	13.5
60	53	Kemali's mixture	26	24

(*c*) *Column Elution*

Elution from carbon can be accomplished on a column if a mixture of 1 part carbon to 2 parts of "Celite Hi Flo" is used instead of pure carbon. Adsorption and washing of the carbon free of salt was done in a flask and then the adsorbant placed in a glass column on top of a few centimeters of "Celite". Twenty-

five grams of carbon with 50 g of "Celite" was placed in a column approximately 50 cm in height and 22 mm in diameter. Five liters of phenol-saturated water was passed through this column in 2 nights and 1 day. The recovery of "aromatic content" based on absorbance measurements was 89% (cf. also Table III(*b*)).

With larger columns, the process of elution took several days or even weeks. In such cases, there appeared to be extensive decomposition of the material on the carbon with melanin formation. Hence, the column technique proved unsuitable for the preparation of extracts from large amounts of urine.

Several attempts were made to fractionate the aromatic materials directly from the carbon by elution from a column with a succession of different solvents. A series of phenol solutions from 0.1% to full saturation were passed in turn through a column of carbon–"Celite" adsorbant mixture and the eluants were evaporated and washed free of phenol; 0.1% phenol failed to elute any appreciable amount of aromatic material and 2–5% eluted the whole spectrum of compounds. No selectivity was observed in the extracts.

In other experiments aimed at combined solvent elution and fractionation a variety of organic solvents were passed through successively. In one series, 95% ethanol was used first, followed by 2% pyridine, and finally by 7% phenol. The ethanol removed 23–28% of the aromatic content as determined by ultraviolet absorbance measurements at 275 mμ, the 2% pyridine removed 14–18%, and the 7% phenol 22–47%. In this, and in the other systems tried, chromatography on the fractions indicated a great deal of overlap and little degree of real fractionation.

3. Use of Other Adsorbants

Von Euler's procedure (13) for the separation of catecholamines was applied to one urine. As anticipated from his reports, the extract contained only a very small amount of material (0.13% of the total aromatic content of the urine is calculated from absorbance measurements). Chromatography of the extract was unsatisfactory because of the high concentration of aluminum salts and the instability of the catecholamines.

Attempts were made to obtain fractions of the aromatic compounds directly from urine by adsorption and elution from ion exchange resins or by precipitation with lead or barium salts; the various extracts obtained from these procedures proved too impure for satisfactory chromatography.

4. Solvent Extraction of Urine

The phenolic acids constituted approximately one-third of the "total phenols" obtained by carbon adsorption and elution, as indicated by the data in Table VI. Because of the effect of one compound on the movement of others in the chromatographic systems, chromatography of these materials was more satisfactory if they were separated from the mixture. The phenolic acids were separated by ethyl acetate extraction of either the crude carbon–phenol extract or of urine itself by Armstrong's procedure (14). Extracting urine gives a fraction which is cleaner for chromatographic purposes than that obtained by extraction of a carbon extract. For this reason we frequently

salt-saturated the urine and extracted it with ethyl acetate before adsorbing it on carbon. Particular care was taken in such cases to wash the carbon free of salt in order to avoid excessive amounts of inorganic materials in the final extract. In two such extracts of urine samples of schizophrenics, 32–35% of the free and 25–27% of the total "phenols" were recovered in the ethyl acetate extracts. Ultraviolet absorbance measurements on two "schizophrenic" and three "normal" urine samples, and on the ethyl acetate extracts obtained therefrom, indicated extraction of 21–25% of the aromatic content in the schizophrenic samples and 10–15% (average 12%) in the normal samples. The carbon–phenol extracts prepared from urine after ethyl acetate extraction still contained a certain percentage of ethyl-acetate-soluble materials which may have either been formed in the phenol extraction procedure or left behind by incomplete ethyl acetate extraction.

n-Butanol extraction of the acidic aqueous residue left after ethyl acetate extraction of urine removed an additional 17% of the "phenols". The extract so obtained had a very high ratio (69:1) of organic solids to free phenols and proved to be rather unsatisfactory for chromatography; this was probably because of the presence of a high proportion of aliphatic constituents since only 5.5% of the total solids were inorganic.

D. Fractionation Procedures

1. Solvent Fractionation

The objective of solvent fractionation of the crude extracts made from urine was to break the extracts into groups of compounds which would resolve well on chromatograms. The most suitable procedure found was to treat the carbon extract with ethyl acetate, followed by methanol–butanol–benzene. Three broad fractions resulted which fell roughly into the R_f regions 0–0.3, 0.3–0.6, and 0.6–1.0 in *n*-butanol/acetic acid/water (4:1:1). Fractionation was followed quantitatively in most instances by measurement of the distribution of organic solids as well as of "phenols".

The standard procedure was as follows: An acidic aqueous solution of parent extract (pH 2) at a concentration of about 5–10 mg/ml of free "phenols" was extracted five times in a separatory funnel with one-half volume of ethyl acetate per extraction. The ethyl acetate extracts were combined, dried, and evaporated under vacuum; the residue was generally taken into water for analytical purposes. Occasionally there was a crystalline residue poorly soluble in water. Isolation and study of such crystals on three occasions showed them to be hippuric acid. Continuous ethyl acetate extraction was tried but there was breakdown of the ethyl-acetate-insoluble materials under the extraction conditions. The ethyl-acetate-soluble fraction was of R_f approximately 0.6–1.0.

The aqueous portion containing ethyl-acetate-insoluble materials was evaporated to dryness, dried by re-evaporation with benzene and then triturated with four portions of methanol/butanol/benzene (2:1:1). This solvent mixture was selected as a result of studying various solvent combinations for their ability to move the polar, ethyl-acetate-insoluble compounds on paper

(see Table V). In the initial trituration the methanol was added first to aid in freeing the solids adhering to the walls of the flask and then the butanol and benzene were added to the solution. The soluble fraction fell in the R_f region of approximately 0.1–0.6. The residue which had an R_f in butanol/acetic acid/water of 0–0.3 was dissolved in water and traces of the solvents evaporated off. In some instances the methanol/butanol/benzene-soluble portion was further subdivided by evaporation to dryness and trituration of the residue with four portions of *n*-butanol. The butanol-insoluble portion was dissolved in water; it had an R_f of 0.1–0.4. The butanol fractions were combined, evaporated to dryness, and the residue taken up in a small amount of water.

TABLE V

Solvent mixtures tried for chromatographic separation of materials of low R_f in butanol/acetic acid/water (4:1:1)

Found unsatisfactory	MeOH/*n*-BuOH/C_6H_6/H_2O (1:2:2:2)
	n-BuOH/NH_4OH/H_2O (100:2:16)
	H_2O saturated with CO_2
	C_6H_6/AcOH/H_2O (2:2:1)
	C_6H_6/12 *N* HCl/H_2O (16:1:8, upper layer)
	$CHCl_3$/HCl/H_2O (20:1:20, lower layer)
	n-BuOH/EtOH/H_2O/NH_4OH (40:10:49:1)
	EtOH/C_5H_5N/C_6H_6 (10:1:5)
	n-BuOH/EtOH/H_2O (4:1:5, upper layer)
	n-BuOH/EtOH/H_2O (4:1:5, lower layer)
	MeOH/C_6H_6/H_2O (2:1:1)
Found possibly satisfactory	MeOH/*n*-BuOH/C_6H_6/H_2O (2:2:1:1)
	MeOH/*n*-BuOH/C_6H_6/H_2O (3:1:1:1)
	MeOH/*n*-BuOH/C_6H_6/H_2O (2:1:1:1)

As indicated by the data in Table VI, this procedure gave reasonably consistent distributions of organic solids from one fractionation series to another if the same type of parent extract were used. For some unknown reason, the schizophrenic extracts seem to contain a higher proportion of ethyl-acetate-extractable organic solids (which do not, however, react in the Folin–Denis procedure for free "phenols") than do the normal extracts and a correspondingly lower proportion of organic solids insoluble in the organic solvents used. Fractionation of one schizophrenic extract made by adsorption on carbon and elution with Kemali's mixture gave the same distribution among the various fractions as did the carbon-phenol schizophrenic extracts. The ratios of total to free "phenols" (about 6 for the normal extracts and 12 for the schizophrenic extracts) were much lower in the ethyl-acetate-soluble fractions than in the parent extracts indicating that, as might be expected, the ethyl-acetate-soluble fraction differs strikingly from the other fractions in that most of its components apparently react as free phenols in the Folin–Denis procedure. Chromatographically there was very little overlap between the ethyl-acetate-soluble and ethyl-acetate-insoluble fractions. The fractions obtained by trituration, on the other hand, all showed some overlap of spots one with another, but the fractions proved more suitable than the parent mixture for further fractionation through ion exchange or other procedures.

Simple extraction of the acidic aqueous residue left after ethyl acetate extraction with a variety of solvents such as isoamyl alcohol, *n*-butanol, benzene, and isobutanol was tried but the degree of fractionation was not as satisfactory as in the procedure described above. Extraction of the ethyl-acetate-insoluble portion with *n*-butanol at pH 8–10 resulted in removal of 30–40% of the ethyl-acetate-insoluble organic solids; such extracts were contaminated with considerable salt (10–15% inorganic solids). Most of the material extracted under alkaline conditions into butanol also went into methanol/butanol/benzene under acidic trituration conditions. An attempt was made to fractionate the methanol/butanol/benzene-insoluble portion by extraction under alkaline conditions with 2% isoamyl alcohol in ethylene dichloride but no material was extracted. Successive trituration of the methanol/butanol/benzene-insoluble residue under acidic conditions with *n*-butanol, ethanol, methanol, and water gave only poor fractionation.

2. Ion Exchange Fractionation

The solvent fractions could be further fractionated by adsorption on ion exchange resins followed by either increment or gradient elution. The first resin used was Dowex-1, an anion exchange resin of the styrene quaternary ammonium type supplied in the chloride form. This resin was converted to its acetate in the usual fashion (15) with sodium acetate, acetic acid, and then water washes. The resin and its acetate were mounted in glass columns 22 mm in diameter. A solvent fraction in aqueous solution was applied to a column containing an estimated 10-fold excess of the resin. Excess solution and unadsorbed material were washed out of the resin with distilled water. The washing was continued until the washings had an optical density of less than 1 ($>$ 10% transmission) when read directly on a DU spectrophotometer at 275 mμ. In increment elution from the acetate resin, the reservoir was filled successively with 0.05 *M*, 0.1 *M*, 0.2 *M*, 1 *M*, and 5 *M* acetic acid. The acetic acid was used as a convenient source of acetate ion which could be removed readily by evaporation. Samples were collected with each eluant until the optical density of the effluent was less than 1. The separate eluants were each evaporated under vacuum and chromatographed as described in the following section.

For gradient elution, a two-bottle reservoir setup was used, arranged so that each portion of eluant contained slightly more acetic acid than the preceding one. Individual 20-ml fractions were examined for ultraviolet absorption, the optical densities plotted against tube number, and the tubes grouped according to the peaks in the plot. The material in each group of tubes was combined, evaporated down, and chromatographed.

The material which was not adsorbed by the Dowex-1 acetate resin was concentrated with the washings for use on the next resin. This was a Dowex-50W cation exchange resin of the styrene – sulphonic acid type, supplied to and used by us in the hydrogen form. It was washed free of ultraviolet-absorbing materials with distilled water. For increment elution from this

TABLE VI

Distribution of material in total extracts among various solvent fractions

	Mean (and range) percentage of parent extracts in terms of:		
	Free "phenols", %	Total "phenols", %	Organic solids, %
Ethyl-acetate-soluble of:			
5 schizophrenic phenol	40 (34–45)	36 (32–43)	29 (23–38)
1 schizophrenic Kemali	—	—	32
3 normal phenol extracts	38 (36–40)	32 (31–33)	14 (11–17)
Ethyl-acetate-insoluble of:			
4 schizophrenic phenol	50 (40–61)	51 (43–61)	67.5 (64–70)
1 schizophrenic Kemali	—	—	66
3 normal phenol extracts	53 (53–53)	64.5 (64–65)	77 (75–79)
MeOH/BuOH/C_6H_6-soluble of:			
5 schizophrenic phenol	31 (27–38)	34 (25–43)	49 (45–57)
1 schizophrenic Kemali	—	—	51
3 normal phenol extracts	31 (30–32)	38 (35–41)	53 (47–64)
MeOH/BuOH/C_6H_6-soluble and BuOH-soluble of:			
4 schizophrenic phenol	17.5 (14–23)	19.4 (14–26)	26.5 (25–29)
1 normal phenol extract	17	19.5	26
MeOH/BuOH/C_6H_6-soluble and BuOH-insoluble of:			
4 schizophrenic phenol	14 (11–15)	14 (11–17)	22 (15–28)
1 normal phenol extract	16	22	21
MeOH/BuOH/C_6H_6-insoluble of:			
5 schizophrenic phenol	9 (5–12)	9 (7–12)	17 (13–19)
1 schizophrenic Kemali	—	—	15
3 normal phenol extracts	18.5 (18–19)	22 (21–23)	27 (25–28.5)

resin, the eluants used were: 1 *M* and 5 *M* acetic acid; 0.1 *M*, 1 *M*, and 6 *M* HCl. This gave graded increments in hydrogen ion concentration. For gradient elution, acetic acid was gradually concentrated as with the anion exchanger and after glacial acetic acid had run through, hydrochloric acid was started and increased up to 6 *N*. Fractions were collected, grouped, and chromatographed as before.

The ion exchange procedure separated the large number of compounds to be found in the various solvent fractions into smaller groups from which a few solid compounds were crystallized. Uric acid, xanthine, 7-methylxanthine, *p*-tyrosine, and ammonium chloride were obtained as crystals from particular fractions and were identified by traditional methods such as melting point, ultraviolet absorption spectra, density, etc.

Quantitative spectral absorbance data showed that there was considerable loss of material on the ion exchange columns. Presumably it either was firmly adsorbed by the aromatic styrene polymer or it melanized to insoluble material under the influence of prolonged exposure to acid.

3. Other Methods of Fractionation

Separation of the crude extract into fractions was attempted through column chromatography on a column of "Celite" and through paper chromatography on Whatman No. 1 and No. 3 MM paper. These methods are generally useful only with already fractionated extracts.

E. Chromatographic Procedures

1. General Procedure

The general procedure for examining the extracts and fractions by two-dimensional paper chromatography was described in a previous paper from this laboratory (5) with some modifications. The amount spotted for each extract or fraction was based in each case on some measure of concentration. In most cases an amount of extract or fraction thereof was spotted which was calculated to correspond to 0.2 mg of Folin–Denis free "phenols" in the parent extract. In some cases the amount spotted was that which corresponded to 5 mg of organic solids. It proved preferable for reproducibility and resolution to spot always on the rough side and to let the solvent run in the machine direction of the paper. In every case descending chromatograms were run on Whatman No. 1, 18½ in. × 22 in. papers, with the basic solvent in the short direction first, followed by acidic solvent in the long direction.

2. Solvent Systems

Our usual solvent systems were isopropanol/ammonia/water (8:1:1) followed by fresh *n*-butanol/acetic acid/water (4:1:1); each solvent was run overnight. The map (Fig. 1) shows the position of the spots where these solvents were used. In some cases, characterization of certain spots was ambiguous if this solvent system were used. The ethyl-acetate-soluble fractions were chromatographed with isopropanol/ammonia/water followed by benzene/propionic acid/water (100:70:5). The spots could then be compared with the maps given by Armstrong *et al.* for phenolic acids (14) and indoles (16). The methanol/butanol/benzene-soluble fractions were studied in our usual solvent system, and in isopropanol/ammonium hydroxide/water followed by *n*-butanol/formic acid/water (77:11:12). This permitted comparison of our purine spots with those reported by Weissman *et al.* (17).

3. Detection Methods

The dried sheets were examined under an ultraviolet light (Mineralite SL 2537). Several phenomenon were observed. First, there was the general background fluorescence in the paper left behind by the solvents. This was particularly noticeable with the benzene-containing solvent which hampered determination of the ultraviolet pattern of such chromatograms. Certain materials such as the purines and hippuric acid appeared as dark spots on the lighter background either because they absorbed the ultraviolet or because they quenched the fluorescence; such spots are labelled "P" in Table VII. Other compounds, such as xanthurenic acid and 2-hydroxyhippuric acid, appeared as definitely colored areas, usually lighter and brighter than the background. Presumably these compounds fluoresced. If the light were rapidly removed, certain spots, as for example kynurenic acid, exhibited phosphorescence; such spots are designated by the symbol (!) beside the color description in Table VII. Chromatograms were examined by transmitted as well as reflected ultraviolet light. Good characterization of the ultraviolet pattern on each chromatogram was essential as a key method of intercomparing chromatograms of the same extract sprayed with different developing reagents.

TABLE VII

Chromatographic characteristics of spots found in urine extracts

Name	Spot No.	$R_f \times 10$ in: BuAc	i Pr Amm	BzPr	UV	DSA	DNA	Alt.	Ehr.	Amm. Ag	B.M.	Nin.	DQC	DQC base	Ag–BPB	Ext. slv.
	118*	1	1			R	—	—	Y	$W^{24}p^{48}$	—	P	—	—	—	W
	230	1	1		—	—	Gy	Y	R^f	Y	Gy^f	M	—	—	—	W
	119*	1	1			R	PiM	—	—	—	—	M	—	—	—	W
(2-Thiol-histidine) (21)	120*	1	1		P	Y	Y	—	—	—	—	W	R^s	—	—	W
	169	1	1		—	Y	Y	—	pY	—	—	P	Pi	Bn		W
	168	1	1		P]	R	W	—	Y	—	—		Pi	Pi	B	W
	121*	1	1		D	YBn	Gy	—	Y	W	Gy	P^{24}	—	—	—	W
	122	1	1		P	Pi	P	—	pY	—	—	M	Y	Y	—	W
	216	1	1		Y	R	Bn	Y	O^{24}	Bn	—	—	—	—	—	W
	215	1	1		—	Pi[P]	b	Y	B	—	—	P^{24}			—	W
	305	1	2		B											W
	113*	1	2		—	Y[P]	Pi[P]		—	—	—	—	B		—	W
	170	1	2		—	—	—	pY^{24}	Y^{24}	Bn^{24}	B	$Bn \rightarrow P$	Gy	Gy	—	W
	105*a**	1	2		d	R	Gy	—	—	—	—	M	—	—	—	W
Histidine (5)	105*b*	1	2		—	R	Ap	pY^{24}	Y	Bn^{48}	—	P	Bn^s	—	—	W
	304	1	2		DB	—	—	—	Y	—	—	—	—	—	—	W
	166	1	5		B	Y	Pi	—	—	—	—	—	—	—	—	W
	184	2	1		D	Y	—	pY	—	W	—	$B \rightarrow P$	OBn	P		W
	156	2	1		B	pPi	—	—	—	$Y \rightarrow Bk$	—	RP	—	—	B	W
Uric acid (21)	123*	2	1	2	P	W[B]	C[Y]	—	—	Bn	—	Bn	Y	O	Gy	EW
	332	2	1		B]	Y	—	—	pYBn	pBn	—	—	—	—	—	W
	157	2	1		D	R	M	—	Y	—	—	P		—	—	W
Uric acid (21)	153	2	1	2	P	W[B	C[Y]	—	—	Bn	—	Bn	Y	O	Gy	EW
	188	2	1			—	—	Pi	—	W	—		—	—		EW
(Allantoin) (21)	934	2	1		—	—	—	—	Y	Pi^{48}	—	—	—	—	—	W
	128*	2	1		d	R	M	O	—	—	Y^a	P	pGy	—	—	W
	158	2	2		dB	C	—	—	Pi^{24}	pBn	—	PiM	—	—	—	W
	217	2	2		d	pPi	Gy	R^{24}	Y	—	—	RP	—	—	—	W
	521	2	2		B	pY	pY	—	bOY	—	B		—	—	—	W
	207	2	2	0	P	—	—	—	$pM \rightarrow B^f$	Gy	—	—	—	—	—	EW
	185	2	2		D	Y[B]	Y[B]	pY	OY	W	P	W	—	—	—	W
	186	2	2		—	Y	Y[B]	—	—	Bn	Y^a		pGy	—	—	W
	†311*a*	2	2		P	pPi	—	—	—	W	—	M	—	—	—	W
	311*b*	2	2		P			O		—	pB					W
	155	2	3		b	RBn	GyP	—	—	—	—	—	—	—	—	W
Ammonium chloride	†104*	2	3	0	—	Y	—	Y	—	Gy	—	—	—	—	Gy	W
	301	2	3		P	pRBn	M	pO	Pi			—	—	—		W
	519	2	4		B	—	—	—	pY	—	P→B	pM	—	—	—	W
	517	2	5		P	—	—	—	Y		—	—	—	—	—	W
	518	2	5		—	—	—	—	pPi^{24}	—	—	—	—	—	—	W
	114	2	5		P	—	—	—	—	—	—	—	—	—	—	W
	115	2	5		—	—	P	—	—	—	—	—	B	—	—	W
	154	3	1		Y	YBn	RP	—	—	—	—	—	P	Gy		W
	124	3	1		—	B	M	—	Y	—	—	M	—	—	—	W

(3-Hydroxy-kynurenine) (19)	522	3	1		Y	Bn	Gy	—	OY	Bn	Pi	Bn	B[f]	—	Bn	W
	187	3	1	0.5	P	pY	—	P	—	W[24]	—	pP	W		—	EW
	129	3	1		DB	YBn	PiB	—	pY	Bn	—	Gy	pY		—	W
	520	3	1		P	R[f]	Gn→OBn	—	pBn	—	—	—	Bn	—	—	W
	526	3	1		—	—	—	—	Gn	—	—	—	—	—	—	W
	213	3	1	0.5	DB	Y	Gy[P]	—	—	—	P	—	R[f]		—	EW
	†112	3	1	1	DB]	Y	Pi	—	OY	pY	—	Gy	Gy		—	EW
	†201	3	1	2	Gn]	Y	Ap	pPi	pO	W[24]→pBn	—	Gy				EW
	302	3	2		P	—	—	—	—	—	—	pB	pB	Gy	—	W
	131*	3	2	0	dB	B	Gy	—	—	Gy[48]	M	M	—	—	—	W
	171	3	2	0	—	YBn	RP	—	—	Gy		M	P[24]		—	W
	174	3	2		P	Y	Ap	O	pY	W	—	Gy	Pi[4]		—	W
	192	3	2		B	R P	M	—	O[c]	YBn[24]	—	—	P	P	—	W
	516	3	2		—	—	—	—	Pi[f]	—	—		—		—	W
	204	3	2		D	Y	Gn→Ap	Pi[24]			P		R[f]	Gy	—	W
Xanthine (21)	111*	3	2		P	OY	pM	—	—	—	—	—	—	M	pB	W
	172	3	2		dB	RBn	Y[Pi]	—	Y	pBn	—	M	—	—	pB	W
	134*	3	2		B	R	M	—	—	—	—	—	B	—	—	W
	130*a*	3	3		b	R	M	—	—	—	—					W
	130*b*	3	3					O								W
	189	3	3					O					B			W
	141*a**	3	3		b	R	M	—	—	—	—	—	—	—	—	EW
	141*b*	3	3					Pi								W
Kynurenine (19)	195	3/4	3	1	B	—	pBn	—	OY	Gy→Bn	P	P	pGy	—	—	W
	102	3	3			OGy										W
p-Tyrosine (21)	103*	3	3		—	RGy	P	—	pY	—	—	P	Bn[2]		—	W
	515	3	3	1	W]	R→GyBn	dGy	—	P→B[e]	—	Y	—	RBn		—	E
	315	3	4		P	—	—	—	—	—	—	—	—	—	—	W
	116	3	6		—	R	P	—	M	—	—	—	Y	—	—	W
	314	4	1		—	Y	Ap									W
Xanthurenic acid (19)	163	4	1	1	W	R[f]	M	—	Y	Bn	—	—	T	B	—	EW
	161	4	2	0	—	R[f]	GyP	—	Pi[f]	—	—	P	RBn	B	—	EW
	338	4	2			Y	Y	—	—	—	—	—	B	P	—	E
	160	4	2		D	RBn	GyP	—	—	—	—	—	—	—	—	W
	133*	4	2		P	W	W	—	—	GyBn	—	—	GnBn	GnBn	Bn	W
(7-Methylxanthine) (17)	179	4	2	5	P	Y[b]	Pi	—	—	R[f]W[24]	—	—	—	B	P	W
	135*a**	4	2		P	LeY	pY[dB]	—	pY	Bk[24]	—	—	Y	YBn	—	W
Hypoxanthine (17, 21)	135*b*	4	2		P	—	—	—	—	—	—	pM[24]	—	Bn	B	W
	132*a**	4	3		dB]	RO	P	—	Y	pBn	—	Ap	Gn[f]		—	EW
	132*b*	4	3		D	PiM	W	—	—	W	—	P	Gy	Y	P	W
	214	4	3		DB	YBn	pM									W
Tryptophane (5)	101*	4	3	2	—	YBn	pBn	—	P→Gn[24]	—	Y[a]	P	pYBn		—	W
(1-Methylhypoxanthine) (17)	136*	4	3		D	bY	Ap	—	—	W	—	—	—	B	P	EW
	513	4	3		D	—	—	—	Pi[24e]	—	—	—	—	—	—	E
	306	4	4	0	P	—	—	—	Pi[f]	—	—	—	—	—	—	W
4-Aminoimidazole-5-carboxamide (21)	159	4	4		P	B[f]	Pi•P	pY	Y	pBn	RP	M	B[f]→YBn	Bn	pB[f]	W
Creatinine	†106	4	4		P	Y	—	pY	—	Bn	–O[d]	—	Bn	Bn	—	W
	109*	4	5	0	—	pY	M	—	RP[e]	Y[24]	—	Y	B		—	W
	307	4	5		—	R	M	—	M	—	—	—	BGn		—	W
(*o*-Tyrosine)	193	4	5	1	—	OY	P	—	pY	Bn[24]	—	B	P		—	W
	228	4	6	0		R	Gy						B			W
	196	4	6		P								Pi			W

TABLE VII (*Continued*)

Chromatographic characteristics of spots found in urine extracts

Name	Spot No.	$R_f \times 10$ in: BuAc	i Pr Amm	BzPr	UV	DSA	DNA	Alt.	Ehr.	Amm. Ag	B.M.	Nin.	DQC	DQC base	Ag-BPB	Ext. slv.
(Indican) (21)	†308	4	6	0	B	Y	—	—	Bn,R[e]	Bk	B					W
	180	4	6	0	Gn	—	pM	—	B[if]	—	—	—	—	—	—	W
	110*	4	6	0	—	OY	RP	—	B[e]	—	—	—	B	B	—	W
	152	5	1		RP	—	—	B		Y→Bn	Y	P	Bn		Y	W
	218	5	1		P	P[f]	P[a],Gy	—	—	Y	Y	M	R→Bn	GyB	—	W
	316	5	2		Pi!	pO	PiM				—	pP				W
	323	5	3		dB	Pi	M	—	Y	—	Y[a]	—	T		—	W
	140*a*	5	3		—	R[b]	Y[dB]	—	—	—		—	Y	Y		W
	140*b*	5	3			R	P		pPi[2d]							W
	183	5	3			R	M			Bn						W
	191	5	3			pPi	B	Pi[f]								W
Kynurenic acid (19)	328	5	3	3	B!	[b]	[b]	—	—	—	—	B[f]	—	—	—	EW
	523	5	3						Pi→M							W
Urea (19)	†501	5	4	6	—	—	—	—	Y	—	—	W	—	—	—	EW
	527	5	4						Pi[dl]			P				E
Phenylalanine (5)	108*	5	5		—	pY	pM	—	pY	W	—	P	Gy→Bn		—	W
	117*	5	7	0	—	OR	P	—	—	—	—	—	Bn	B	—	W
	310	5	7	0	dB	—	—	—	Y	—	—	—				W
	203	6	1	2	P	pPi	W	P	—	Gy	—	—	Bn[3d]	—	—	EW
	512	6	1						Y							EW
	317	6	2		B			pPi								EW
	164	6	2		—	YBn	Pi	—	—	—	—	—	—	B	—	W
	511	6	2						Y							EW
Urocanic acid (5)	107*a**	6	3	6	P	RO[RBn]	P	—	—	—	pBn	—	Bn	Bn	—	EW
(1-Methylxanthine) (17)	107*b*	6	3	4	P	R	P	—	—	R[i]→W	—	—	Gy	Gy→P	B	W
	167	6	3		D	pPi	PiM						C			EW
	202	6	3		dB	B	B[B]			Gy→Bn						W
(4-Hydroxy-3-methoxymandelic acid) (14)	10*	6	3	3	D	RBn	P	—	—	Gy	—	—	GyB	B	—	E
	529	6	3	4		pBn	—	—	Pi[if]	pBn	—	—	—	—	—	E
(5-Hydroxyindole-3-carboxylic acid) (16)	325	6	3	4					Pi[i]→B							E
	324	6	3		D	Y[b]	R									E
	333	6	3		dB	pOY	R[a]	—	—	—	—	pM	B	B	—	W
Adenine (17, 21)	318	6	4		P	pY	—	—	—	—	—	—	—	—	dB	W
	326	6	4		BGn	—	—	—	—	—	—	—	—	—	—	EW
	†309	6	6	7	P	—	—	—	—	—	—	—	—	—	—	EW
	334	6	6		—	Y	Y	—	RBn	—	—	—	—	—	—	W
	215	6	6	2	—	Y	R	—	pY	—	—	—	—	—	—	W
	223	6	6		—	Y	Pi	Y	Y	—	—	—	—	—	—	W
	224	6	6		—	Y	Pi	—	—	—	—	—	—	—	—	W
	225	6	7		—	Pi	M	—	—	—	—	—	—	—	—	W
	165	6	8			Pi										EW
	175	7	2	3	—	pPi	P	—	—	—	Y[a]	—	pPi	pB	—	E
	219	7	2		P	—	PiM[b]	O	P[f]	Pi	—	—	—		—	E
(4-Hydroxy-3-methoxy-cinnamoylglycine) (14)	16*	7	2	3	dB	P[f]	GyB	Ap	—	pBn	—	—	GyB		—	E

(4-Hydroxy-3-methoxyhippuric acid) (14)	17*	7	2	3	d	P→R	M	O	—	—	—	—	pB		—	E
5-Hydroxyindole-3-acetic acid (16)	5*	7	2	3	b	RBn[f]	Pi[a]M	—	B[a]	Gy	O[a]Bn	RBn	Pi	P	Bn	E
(3-Hydroxyhippuric acid) (14)	3*	7	3	3	dB	OY	RP	O	—	—	—	—	B	T	—	EW
	327	7	3		dB	PiM[b]	M	Ap	Y	—	Y	pM	bB		—	E
(4-Hydroxymandelic acid) (14)	9*	7	3	2	—	C	Pi	—	—	—	—	—	—	B	—	E
(3-Hydroxy-3-(4′-hydroxy-3′-methoxyphenyl) propionic acid) (24)	18*	7	3	4	—	OR	P		—	Gy	—	—			—	E
	24	7	3	5	—	Y	RP	—	Pi[f]	—	—	—				E
	502	7	3	4–6	d	Y	M	—	Pi[a]	—	—	—				E
2-Aminohippuric acid (14)	52*	7	3	3	B	Y	Y	O	Y	—	P	—	B[24]		—	E
(4-hydroxyphenyllactic acid) (14)	14*	7	4	2	—	RP	P	—	—	YBn	—	—	—	—	—	E
	211	7	5					Pi[24]	pPi							W
	222	7	6		—	Ap	O	—	—	—	—	—	pGy		—	W
	508	7	6		—	RGy	M	—	bY	Gy	—	—	Gy[24]	B	—	EW
	319	7	7		dB	—	—	—	—	—	—	—	—	—	—	E
	320	7	7		Y	—	—	—	—	—	—	—	—	—	—	E
	178	8	1	4	!	RGy	pM	—	—	pGy[24]	—	—	—		—	E
4-Hydroxyhippuric acid (14)	† 4*	8	2	2	P	pO	Pi	O	—	—	—	—	—		—	E
	181	8	2		—	pBn	YBn	—	Pf	Bn					—	E
	151	8	2		P!	—	M→B	Pi	—	Gy	—	—			—	E
3,5-Dihydroxybenzoic acid (14)	23	8	2	6	—	O	pBn	—	—	Bn	—	—	M	P	—	E
2-Hydroxyhippuric acid (14)	† 6*	8	2	5	B	bY	R	O	—	pGy	—	—	P	BT	—	E
	227	8	3		Y	pY	OY	—	—	—	—	—	M		—	E
	150	8	3		R	pY	pY	Pi	—	pY	Y[a]	—	pY[f24]		—	E
	220	8	3	8	—	—	—	—	Gn[24]	—	—	—	—		—	E
	221	8	3		P	Bn	P	Pi	Gy[24]	Bn	Ap[a]	—	M		—	E
4-Hydroxy-3-methoxy phenylacetic acid (14)	7*	8	3	7	b	R	GyB	—	—	Gy	Y[a]	—	Bn	BBn	—	E
4-Hydroxyphenylacetic acid (14)	1*	8	3	6	—	R	P	—	—	—	—	—	pBn		—	E
(3-Hydroxy-3(3′-hydroxyphenyl) propionic acid) (14)	2*	8	4	3	—	OY	M	—	—	YBn[24]		—	P	B	—	EW
	210	8	4	5	—	pY	—	Y	Y[a]	Y[24]		W	Bn	—	—	E
	182	8	4		B	—	—	—	—	—	—	—	—	—	—	E
	506	8	4	4	P	pY	—	—	P→Gy	—	Y[a]	—	—	—	—	E
N-acetyl-tryptophan (16)	66*	8	5		dB	pY	pPi	—	P→GyB[24]	Bn[dl]	—	—	Bn[dl]	—	—	E
	176	8	5	5	B	Y	Pi	—	Pi[f]	—	Gy[24]	—	—	B	—	E
Hippuric acid (19)	† 53*	8	5	6	P	—	—	O	—	—	—	—	OBn	Bn	—	EW
	229	8	6		D	—	—	—	Pi[df]	—	—	—	—	—	—	E
	322	8	7						Pi→B							E
	321	8	8		Y				Pi[df]							E
	212	9	1	5	Y	—	—	—	—	—	—	—	—	—	—	E
	208	9	1	7	P	—	—	O	pRGy	W[24]	—	—	—	—	—	E
(Indoleacetyl glutamic acid) (16)	209	9	1	3	—	pRGy	—	pO[24]	P[f]pBn	Gy	—	—	—	—	—	E
	166	9	1	1				Pi	Pi[24]							E
4-Hydroxy-3-methoxybenzoic acid (14)	19*	9	2	8	d!	O	P	—	—	Bk	Y[a]	—	M	B	—	E
4-Hydroxybenzoic acid (14)	12*	9	2	6	P	Y	R	—	—	—	—	—	B	B	—	E
4-Hydroxycinnamic acid (14)	33	9	3	7	D	R	GyB	—	—	Bn	—	pBn	Bn	Bn	—	E
	329	9	3		—	—	—	Pi	—	—	—	—	—	—	—	E
	505	9	3	7	—	—	—	—	M[24a]	—	—	—	—	—	—	E
3-Hydroxybenzoic acid (14)	15*	9	3	7	B	Y	RP	—	—	—	—	—	B	B	—	E

TABLE VII (*Concluded*)

Chromatographic characteristics of spots found in urine extracts

Name	Spot No.	$R_f \times 10$ in: BuAc	i Pr Amm	BzPr	UV	DSA	DNA	Alt.	Ehr.	Amm. Ag	B.M.	Nin.	DQC	DQC base	Ag–BPB	Ext. slv.
4-Hydroxy-3-methoxyphenyl propionic acid	20*	9	3		—	PiM	GyB	—	—	Bn	—	—	M	Bn	—	EW
	525		3	7	d	—	—	—	Pi[f]	—	—	—	—	—	—	E
(3-Hydroxyphenylacetic acid) (14)	11*	9	4	6	—	O	M	—	—	—		—	RP		—	E
Indole-3-acetic acid (19)	504	9	4	8	dB	YBn[b]	Pi[a]RBn	—	GyB[24]	—	—	—	Bn[24]		—	E
2-Aminobenzoic acid (19)	51*	9	4	8	B	Y	Y→Pi[12]		OY	pBn	B	Y	BGn	B	—	E
	330	9	4		O					Gy						W
	199	9	4	7	—	—	—	Y	B[1]	Gy	—	—	GyB		—	E
	336	9	5	7	O	Y	Y	Y	O	Y	—	—	B		—	E
(3-Hydroxyphenylpropionic acid) (14)	21*	9	5	7	—	O	P	PY	Y				M	B		E
	503	9	5	7	d	—	—	Y	P[f]	Gy[24]	Gy[24]	—	B		—	E
	197	9	5		—	OY	P	—	Y	—	—	—	pB	B	—	E
	335	9	6		—	Gn[f]	P[a]	—	OY	—	—	—	—	—	—	W
2-Hydroxybenzoic acid (14)	27*	9	6	8	B	pY	pO	—	—	—	—	—	B	B	—	E
2-Hydroxyphenylacetic acid (14)	13*	9	6	7	—	O	P	—	—	—	—	—	B	B	—	E
2-Acetamidobenzoic acid (5)	56*	9	6		B!	C	C	—	OY	—	—	—	—	—	—	E
	323	9	6	9	P	—	—	—	—	—	—	—	—	—	—	E
	528	9	6	8	—	—	—	—	Pi[24s]	—	—	—	—	—	—	W
	162	9	7		D	O	pY	—	—	Bn	Y	—	MBn	—	—	E
	303	9	8		Y											E
	313	9	8		D	pPi	Gy	Y[24]	Y[24]	—	—	—	GyB			E
	206	9	9	9	dB	Y	R	—	—	—	M	—	B	B	—	E

NUMBERS: * indicates spots reported in Acheson (5). The authors apologize for the fact that numbers used for some spots (unstarred), while the same as in the previous paper, do not necessarily correspond. They definitely agree with Smith's declaration that any numbering system begins as a chaos and despite revisions appears at some later date as a new chaos.

† indicates spots useful as markers.

SOLVENTS: BuAc = *n*-BuOH: HOAC: H_2O (4:1:1); iPrAmm = *i*-PrOH: NH_4OH: H_2O (8:1:1); BzPr = benzene: propionic acid: H_2O (100:70:5).

UV: !, phosphorescence; b, weak blue; B, sky blue; dB, intense sky blue; DB, blue, darker than background; P, purple, darker than background; Y, yellow; Gn, green; W, white; Pi, pink; O, orange.

SPRAYS: DSA, diazotized sulphanilic acid; DNA, diazotized *p*-nitroaniline; Alt, Altman (PDAB in acetic anhydride); Ehr., Ehrlich's reagent (PDAB in ethanol and HCl); Amm. Ag, ammoniacal silver nitrate; B.M., Bratton Marshall (20) (nitrous acid, then coupling component); Nin., ninhydrin; Ag–BPB, silver nitrate plus bromphenol blue; DQC, 2,6-dichloroquinone chloroimide; DQC base, saturated Borax sprayed over DQC.

COLORS: Colors are defined in Table IV. Superscripts: numbers indicate the time in hours for the appearance of the color; i, immediate; a, color after nitrite spray of DNA and BM.; c, color after spraying with sodium acetate on top of Ehrlich's; d, color with picric acid spray; s, stable; f, fades. Adjectives: p, pale; b, bright; d, dark.

— indicates no reaction while a blank indicates undetermined.

[ ] enclosing a color symbol indicates the color which appeared under ultraviolet after the spray.

Ext. slv.: Extraction solvents: W, aqueous fraction; E, ethyl acetate fraction.

Nine spray reagents were used to characterize the spots as indicated in Table VII. The diazotized sulphanilic acid (DSA), diazotized *p*-nitroaniline (DNA), silver nitrate, ninhydrin, and Altman reagents were prepared and used as described previously (5), but the Ehrlich spray was modified to 2 g of *p*-dimethylaminobenzaldehyde, 80 ml of 95% ethanol, and 20 ml of concentrated hydrochloric acid. The Eckman reagent, 1-ethylaminonaphthalene, for diazotizable aromatic amines which was used previously (18,19), was replaced by the N-(1-naphthyl)-ethylenediamine used in the Bratton–Marshall reaction (20). For this reaction, the dried chromatogram was sprayed with a freshly mixed solution of 1% sodium nitrite 1 *N* and 0.2 *N* sulphuric acid and was allowed to stand for 5 minutes. It was then sprayed with 5% ammonium sulphamate and allowed to stand. Finally, it was sprayed lightly with 1% N-(1-naphthyl)-ethylenediamine and allowed to develop at room temperature. Magenta to blue spots appeared on a colorless background. The spot colors tended to fade and the background turned pale yellow-green after several days.

The bromophenol blue and the 2,6-dichloroquinone chlorimide (DQC) reagents were the standard preparations described in the recent book by Smith (21) except that the reagents were sprayed on.

With both the DSA and DNA sprays, some colors came up and faded very rapidly so that careful watching during and immediately after spraying proved rewarding. Certain spots showed pronounced fluorescence under ultraviolet light after reaction with DSA or DNA sprays; this is indicated in Table VII by the enclosure of the color description for the particular spray in brackets.

Sequential spraying of the same paper with a number of sprays, as recommended by Smith (21), was used occasionally but was not found particularly helpful.

4. Color Description

The color definitions are given in the customary vague descriptive terms. An attempt is made, however, in Table VIII to improve on this classical method by providing an approximate range according to the Color Harmony Manual scale (22) for each color described. Chromatographic colors vary considerably so that such exact color definitions as are possible with the Color Harmony Manual are overprecise; nevertheless, it is felt that much subjectivity can be removed by giving more exact data on chromatographic colors than simple word descriptions.

Results

The data in Table VII comprise our best present characterization of the over 200 spots we have seen in chromatograms of over 70 urine extracts and fractions thereof. Spots seen only very occasionally have been omitted since they may reflect rare dietary constituents. The table lists the spots by number and where possible by name. Because of their greater reproducibility, the positions of the various spots relative to one another, as indicated on the map (Fig. 1), are of more value than the R_f's. For the purpose of reporting the many compounds, the map has been divided up into areas of 0.1 R_f unit in

each dimension. The figure quoted for each spot in Table VII refers not to its exact R_f, which is doubtful, but to the upper limits of the area in which it appears on the map. It may run to as much as 0.1 unit less in each dimension of the chromatograms. Certain spots have proved to be useful as markers; these have been indicated by shading on the map and by a dagger (†) in Table VII. The colors with ultraviolet light and with nine sprays, and the approximate characteristics of fractionation for each spot are given. A (–) indicates that there is no visible reaction with a particular spray while a blank indicates that the reaction, if any, has not been determined.

TABLE VIII

Color description correlated with Ostwald color solid definition (according to Color Harmony Manual (22))

Color description used here	Abbreviation in Table VII	Color definition		
		Hue No. (color chip No.)	Amount of: White	Amount of: Black
White	W	—	*a*	*a*
Black	Bk	—	*p*	*p*
Gray	Gy	—	*g–l*	*g–l*
Brown	Bn	4	*l–p*	*e–g*
Lemon yellow	LeY	1	*e–p*	*a*
Yellow	Y	2	*l–p*	*a*
Green brown	GnBn	2	*n–p*	*e–g*
Cream	C	3	*e–p*	*a*
Orange yellow	OY	4	*l–p*	*a*
Yellow brown	YBn	4	*l–p*	*c–g*
Blue brown	BBn	4	*i*	*g*
Orange	O	5	*i–p*	*a*
Orange brown	OBn	5	*i–p*	*c–e*
Gray brown	GyBn	5	*g*	*e*
Orange red	OR	6	*i–p*	*a–c*
Red orange	RO	6	*l–p*	*e*
Red brown	RBn	6	*n–p*	*e–g*
Apricot	Ap	6	*i–l*	*c*
Orange gray	OGy	6	*i*	*c–e*
Pink	Pi	7	*e–i*	*a*
Red	R	7	*l–p*	*a*
Red gray	RGy	7	*i–l*	*c*
Mauve brown	MBn	7	*i*	*e*
Pink mauve	PiM	8	*i–p*	*a–c*
Mauve	M	9	*i–p*	*a–c*
Gray mauve	GyM	9	*i–n*	*e–i*
Red purple	RP	10	*l–p*	*a–c*
Purple	P	11–12	*c–p*	*a–e*
Gray purple	GyP	12	*g–p*	*g–i*
Blue	B	13–14 15		
Gray blue	GyB	13–14	*g–p*	*c–i*
Blue turquoise	BT	16		
Turquoise	T	17		
Blue green	BGn	18		
Green	Gn	19–24		

NOTE: As can be seen from this Table, the scale of white is alphabetic from *a* (all white) to *p* (no white) in the order of decreasing amounts of white; while the complementary scale of blackness is reverse alphabetic from *p* (all black) to *a* (no black) in the order of decreasing amounts of black.
Only alternate letters are used in the Color Harmony Manual (22).

Names have been given to spots which have been positively, probably, or tentatively identified. Where the identification is positive the name in Table

VII has been italicized. This means that the solid material has been isolated from the urine extracts, purified, and characterized by appropriate methods, and that this isolated material corresponds to the indicated spot. The only exception is 4-aminoimidazole-5-carboxamide (spot No. 159), which was not isolated in solid form from urine but was identified by comparison of spot No. 159 eluted from a chromatogram with synthetic 4-amino-5-imidazolecarboxamide in terms of (*a*) ultraviolet absorption spectra, (*b*) spectra of the Bratton Marshall reaction products, (*c*) identity of the color reactions with DSA, DNA, Ehrlich, Altman, ninhydrin, silver nitrate spray reagents and under ultraviolet light, and (*d*) identity of R_f's when run as parallel unidimensional chromatograms in various solvents (Table IX).

Where the identification is probable, the name of the compound is not italicized. This means that the compound has not been isolated as such from urine but that there is chromatographic correspondence between the standard and the given spot. Such correspondence was determined by running the standard with a sample of extract two-dimensionally and observing whether or not there was augmentation of the spot in all cases with all sprays sensitive to that spot. As a further check, the standard was also run unidimensionally in each of the solvents on the same paper.

Where identification is tentative, the name of the compound is given in parentheses. This means that the standard was not available to us but the spot corresponds in R_f's and color reactions to those reported for that compound in the literature.

TABLE IX

Solvents used for comparative chromatography of No. 159 and synthetic 4-amino-5-imidazolecarboxamide

Solvent	R_f of both
n-BuOH/AcOH/H_2O (4:1:1)	0.4
i-PrOH/NH_4OH/H_2O (8:1:1)	0.4
n-BuOH/EtOH/H_2O (4:1:1)	0.4
n-BuOH/AcOH/Pyridine/H_2O (4:1:1:2)	0.5
2,6-Lutidine/H_2O (65:35)	0.65
BuOH saturated with H_2O + a few drops of HCOOH	0.0

It must be remembered that the chromatographic behavior and even color reactions of a compound are influenced by their concentration relative to the concentration and nature of other compounds in the sample. A given compound may even split into two spots on a single chromatogram or may show different R_f values in a fraction as compared with the parent extract. There is, furthermore, always some partition between fractions. For these reasons, two spots shown in Fig. 1 and described in Table VII may, in some instances, correspond to a single compound. An example is uric acid which may show up as both spots No. 123 and No. 153 or only as spot No. 153, depending upon its concentration; this effect has been noticed before with creatinine (23). On the other hand, several compounds may overlap each other and not be differentiated even by the various fractionation techniques we have used; in such

instances the spot characterization given here may eventually prove to correspond to a number of chemically distinct materials. In cases where such overlapping of spots has been recognized, the spot is described as *a* and *b*. Thus, for example, No. 107*a* is urocanic acid and No. 107*b* is tentatively identified as l-methylxanthine; these two spots which overlap in our usual solvent systems can be separated in butanol/pyridine/water (140: 30: 30).

Discussion

The over-all map shown in Fig. 1 represents, we believe, a fairly complete summary of water-soluble aromatic products in urine extracts obtainable through the use of our techniques. Although these are not necessarily all identical with the aromatic materials occurring in urine because of possible degradation during work-up, the report of Asatoor and Dalgliesh (9) and trials of the carbon–phenol extraction procedure with several pure compounds, such as phenylalanine, tyrosine, histidine, and tryptophan, suggest that many aromatic materials in urine will come through the extraction procedure unchanged.

There are a number of sources of the aromatic compounds in these urine extracts. Many arise through metabolism of the aromatic amino acids, phenylalanine, tryptophan, and histidine. Metabolites in purine synthesis and breakdown are also present and, possibly, some of the pyrroles formed in porphyrin metabolism. Some of the compounds, particularly in the ethyl acetate fraction, are known to arise partly from aromatic materials in vegetables and other dietary products (14, 19, 24). This was demonstrated to us in two experiments. In one, samples from persons on a diet containing only steak, bread, and honey showed a marked reduction in spots of known exogenous phenolic acids and some increase in presumed imidazole spots. In a second experiment, a 3-day diet lacking coffee, tea, and cocoa resulted in a urine sample whose extract showed none of the catabolites of the methylated purines, caffeine, theophylline, and theobromine.

The only chemical identification which seems worth special comment is that of 4-aminoimidazole-5-carboxamide (spot No. 159). A Russian paper (25) reporting this material as a constituent of human urine came to our notice only very recently and to our knowledge was the first recognition of its presence in urine. The compound is well known, of course, as a by product of purine metabolism in sulphonamide-inhibited bacterial cultures and in some bacterial mutants and fungi.

The differences found between the schizophrenic and normal extracts have not been stressed. Difficulties in quantitation and the uncertain origin of many of the spots, coupled with the relatively small number of extracts studied, make us feel that significant conclusions can be drawn only after further work.

Summary

1. A comprehensive two-dimensional "chromatographic map" of over 200 "aromatic" spots found in extracts of human urine is presented.

2. Pools of both schizophrenic and normal urine were examined, but due to the relatively small number of extracts studied and the uncertain source of most of the compounds, differences between the two types are not stressed.

3. It was necessary to extract the crude urine to separate the many compounds by chromatography. This was done by adsorption on charcoal followed by elution with phenol-saturated water and by solvent extraction.

4. The extracts were further fractionated by solvent extraction and through the use of ion exchange resins on columns.

5. Extracts and fractions were chromatographed using isopropanol/ammonia/water and butanol/acetic acid/water as the principal solvent systems.

6. Spots on chromatograms were categorized using ultraviolet light and nine spray reagents as developers.

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SOME STUDIES ON TRANSAMINATION WITH OXALOACETATE[1]

G. Y. N. Iyer and M. Sukumaran

Abstract

The formation of alanine in the transamination of α-amino-*n*-butyrate and glutamate with oxaloacetate in presence of liver homogenates has been studied. It could be quantitatively explained in terms of pyruvate formed by the decarboxylation of oxaloacetate and subsequent transamination. Pyruvate at high concentrations was found to have an inhibitory effect on the α-aminobutyric–pyruvic transamination. The transaminase activity of the homogenate with respect to the glutamic–oxaloacetic system was very marked whereas it was negligible in the case of the α-aminobutyric–oxaloacetic system. In the light of these findings the relative yields of alanine from the two systems are correlated.

Introduction

In the course of a study of the transamination reaction between α-amino-*n*-butyric acid (ABA) and oxaloacetate (OA) in presence of rat liver homogenate we observed (1) the formation of appreciable amounts of alanine. Rowsell (2) has reported that in transamination reactions in which OA is the amino acceptor, some alanine is formed. This author has suggested that OA undergoes β-decarboxylation to pyruvate which then transaminates with the amino acid to yield alanine. On the basis of this explanation the formation of alanine observed by us can be qualitatively explained in view of the fact that ABA–pyruvic transaminase has been demonstrated in liver homogenate (1).

It has also been noted (1) that incubation of ABA with OA in presence of liver homogenates yielded larger amounts of alanine than of ABA with pyruvate at identical concentration. But when glutamic acid was used in place of ABA the amount of alanine formed from OA was always less than that from pyruvate. Consequently the ratio of the amount of alanine formed in incubation with OA to that formed in incubation with pyruvate is distinctly higher when ABA is used as amino donor than when glutamate is used. It was the object of the present investigation to study how far these findings could be quantitatively explained by the pathway postulated by Rowsell (2). In this connection the rate of OA decomposition in presence of rat liver homogenate was followed and the alanine formation studied in two parallel systems having OA as amino acceptor in one and, in the other, pyruvate, added at the same rate as it was presumably formed from OA. With ABA as amino donor equivalent amounts of alanine were found to be formed in the two systems, thereby establishing the adequacy of Rowsell's explanation.

But two facts had still to be explained, viz. (i) that ABA–OA system produced more alanine than the ABA–pyruvate system when the OA and pyruvate were taken at equal concentrations and (ii) that the situation was reversed using glutamate in place of ABA. In order to gain an insight into the first aspect we considered it necessary to study the effect of pyruvate concentration on the yield of alanine in the ABA–pyruvate reaction. For the interpretation

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Contribution from the Department of Biochemistry, Medical College, Trivandrum, India.

of the second aspect the balance sheet of OA, pyruvate, aspartate, and alanine in the glutamic–OA system had to be drawn up. The results of these investigations are presented in this communication.

Materials and Methods

1. Reagents

(*i*) *Substrates.*—ABA (0.1 *M*), glutamate (0.1 *M*), pyruvate (0.07 *M*), and OA (0.07 *M*) were prepared as described in the previous communication (1) but in water in place of phosphate buffer. In the preparation of OA particular care was taken to cool the solution in ice during neutralization with alkali.

(*ii*) *Aniline citrate reagent.*—Five grams of citric acid was dissolved in 5 ml of water and 5 ml of freshly distilled aniline added. The reagent was prepared on the day of its use.

2. Tissue Homogenate

Freshly dissected liver from adult albino rats (either sex) was wiped free of blood with filter paper and homogenized in ice-cold distilled water (20 ml/g of wet tissue) using a tissue grinder with a Teflon pestle.

3. Decarboxylation of OA

The rate of decarboxylation of OA in presence of liver homogenate was studied as follows:

Eight milliliters of homogenate, 1 ml of OA, and 1 ml of water were mixed and incubated at 38° C. The OA content of the reaction mixture at 15, 30, 60, and 90 minutes was estimated by a modification of the manometric method of Umbreit *et al.* (3). One milliliter of the reaction mixture was mixed with 1 ml of 0.4 *N* HCl kept chilled in ice; 1.5 ml of the resulting mixture was pipetted into the main chamber of a 15-ml Warburg flask; 0.5 ml of aniline citrate reagent was taken in the side arm. After equilibration for 10 minutes in the bath (at 38° C) the aniline citrate was tipped into the main compartment and the volume of CO_2 evolved measured. Simultaneously a 'blank' experiment was conducted where the homogenate (8 ml plus 2 ml water) was incubated under the same conditions as the 'test' and 1-ml aliquots subjected to the same procedure. This would enable corrections to be applied for volume changes due to mixing of the contents of the main chamber and the side arm, and also for any small amount of acetoacetate or bicarbonate in the homogenate. The CO_2 evolved in the 'test' in excess of that in the 'blank' was found and the OA content of the reaction mixture at different intervals calculated. The OA assays by this method gave values 5% lower than the theoretical, and the corrected values have been reported.

4. Pyruvate Estimation

The estimations of pyruvate were carried out by the method of Friedemann and Haugen (4). Since pyruvate occurred along with OA, the latter also had to be converted to pyruvate, and the total pyruvate estimated. The OA in the same sample was determined manometrically and the actual amount of pyruvate calculated by difference. One milliliter of the reaction mixture was added with shaking to 5 ml of 10% w/v trichloroacetic acid in a 25-ml measur-

ing flask. After 10 minutes the volume was made up to the mark with water and filtered. To 1 ml of the filtrate 1 ml of 0.8 *N* HCl was added and the mixture heated in a boiling water bath for 10 minutes. This would convert the OA to pyruvate. After the mixture was cooled to room temperature 2 ml of 2,4-dinitrophenylhydrazine (0.1% w/v in 2 *N* HCl) was added and allowed to stand for 10 minutes. The dinitrophenylhydrazone was extracted with 5 ml of benzene and all the subsequent steps were exactly as described by Friedemann and Haugen (4).

5. Transaminase Activity

The method of studying transaminase activity was similar to the one previously described (1). Four milliliters of the homogenate was incubated for 1 hour with 0.5 ml of 0.1 *M* amino acid and 0.5 ml of 0.07 *M* keto acid equivalent to 35 μmoles. The resulting amino acid was estimated.

6. Experimental Verification of Rowsell's Theory as Applied to the ABA–OA System

The rate of pyruvate formation by the decomposition of OA was determined as described above. With minimum loss of time 4-ml aliquots of the homogenate were pipetted into four 25-ml measuring flasks A, B, C, and D. To A were added 0.5 ml of ABA and 0.5 ml of OA, to B 0.5 ml of OA, and to C 0.5 ml of ABA. The four flasks were incubated at 38° C. At 7.5-minute intervals an appropriate volume of 0.04 *M* pyruvate solution was added to C and D so as to match the rate of pyruvate accumulation already determined. One hour after the beginning of the incubation A and B were removed from the bath, 0.5 ml of ABA added to B, and the mixture deproteinized by the prompt addition of 10 ml of acetone. Likewise 1 hour after the beginning of pyruvate addition, C and D were treated exactly as A and B respectively. The alanine formed in A in excess of B, and in C in excess of D, was subsequently determined.

Results and Discussion

ABA–OA System

It can be seen from Table I that incubation of tissue homogenates with ABA and OA (35 μmoles)* results in the formation of larger amounts of alanine than with ABA and pyruvate (35 μmoles). This is in agreement with our previous observation (1) in spite of the fact that we have homogenized the tissue in water in place of phosphate buffer.

Instead of using 35 μmoles pyruvate in the ABA–pyruvate reaction, pyruvate was added at a rate at which it was formed from OA (Table II and Fig. 2), under identical conditions, thereby simulating the ABA–OA system; the yield of alanine was then very nearly equal to that from the ABA–OA system itself (Table I). This in effect establishes quantitatively the adequacy of Rowsell's theory.

But it was still difficult to explain how the ABA–OA system could form more alanine than the ABA–pyruvate system when both the keto acids were

*The concentration of α-keto acids expressed in micromoles represents the quantity in 5 ml of the reaction mixture.

TABLE I

Alanine formation in transamination of α-amino-*n*-butyric acid/glutamate with oxaloacetate/pyruvate

Expt. No.	Alanine formed (μmoles/g of tissue/hour) by transamination between:					
	ABA and 35 μmoles OA	ABA and 35 μmoles pyruvate	ABA and pyruvate (0→23 μmoles*)	Glutamate and 35 μmoles OA	Glutamate and 35 μmoles pyruvate	Glutamate and pyruvate (0→23 μmoles*)
1	6.3	4.2	6.0	5.0	19.5	9.5
2	5.1	3.1	4.9	3.2	18.0	11.4
3	5.2	3.3	5.0	3.0	21.5	6.7

*Represents the rate of pyruvate formation by OA decomposition in presence of homogenate.

TABLE II

Oxaloacetate decomposition and pyruvate formation in presence of liver homogenate with and without added glutamate

Expt. No.	In presence of glutamate				In absence of glutamate			
	15 min	30 min	60 min	90 min	15 min	30 min	60 min	90 min
	OA disappeared, μmoles							
1	19.0	31.5	32.5	34.0	9.5	17.0	27.0	29.0
2	15.0	27.0	31.0	32.0	8.0	15.0	24.5	26.0
3	20.0	30.0	33.5	34.0	12.0	20.0	31.5	33.0
	Pyruvate formed, μmoles							
1	4.0	9.8	10.8	10.8	8.5	15.3	23.0	25.0
2	3.0	6.0	8.7	8.8	6.0	12.0	20.5	21.0
3	3.5	8.0	10.5	10.5	9.0	16.0	26.0	28.0

NOTE: Initial concentration of OA = 35 μmoles/5ml of reaction mixture.

taken at identical initial concentration. If we assume the *de novo* formation of pyruvate from OA as an essential intermediate step it would seem paradoxical that the former system, where only smaller amounts of pyruvate are formed by the decomposition of OA, should yield larger amounts of alanine than the latter where pyruvate is added, as such, right from the beginning of the incubation in larger amounts. What one would normally expect is that with increasing availability of pyruvate the alanine yield would increase up to a point and then remain practically stationary. The observation at hand therefore suggests an unusual effect of pyruvate concentration on the alanine yield in the ABA–pyruvate system.

Further study of this aspect yielded some interesting results which are summarized in Fig. 1. It could be seen from this graph that with increase in pyruvate concentration up to about 14 μmoles the yield of alanine also increases, beyond which the relation is reversed.

The progressive diminution of alanine formation with rise in pyruvate concentration above 14 μmoles may be a case of "substrate inhibition" of the ABA–pyruvate transaminase by pyruvate. It may be recalled (5) that in the case of the enzymatic reduction of pyruvate by lactic dehydrogenase of rabbit erythrocytes, pyruvate has been shown to have an inhibitory effect at high concentrations. Numerous examples of this type of substrate inhibition have been described and various explanatory hypotheses suggested (6, 7). The

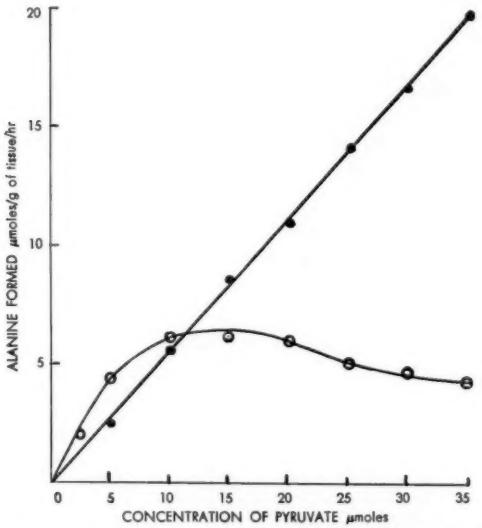

FIG. 1. Effect of pyruvate concentration on the alanine yield in the ABA–pyruvic and glutamic–pyruvic transaminase systems.

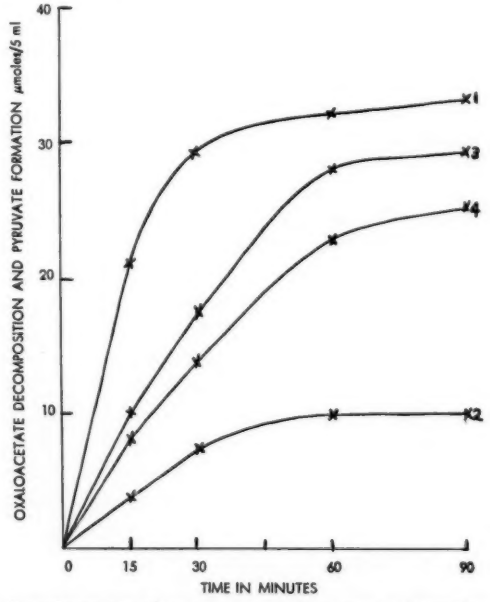

FIG. 2. Rate of oxaloacetate disappearance and pyruvate formation in presence and absence of added glutamate.

1. OA disappearance in presence of glutamate.
2. Pyruvate formed in presence of glutamate.
3. OA disappearance in absence of added glutamate.
4. Pyruvate formed in absence of added glutamate.

nature of the mechanism operative in the case in point will have to be further investigated.

The rate of disappearance of OA and of the consequent formation of pyruvate in presence of homogenate are shown in Table II and Fig. 2. It could be seen that the pyruvate concentration reaches a value of 5 μmoles in about 10 minutes after which it gradually increases to about 23 μmoles in 1 hour. Reference to Fig. 1 would show that after the first 10 minutes the pyruvate concentration attained at any instant during the period of incubation would be much more favorable for alanine formation than a level of 35 μmoles. These relations are quantitatively expressed in Fig. 3 where the rate of alanine for-

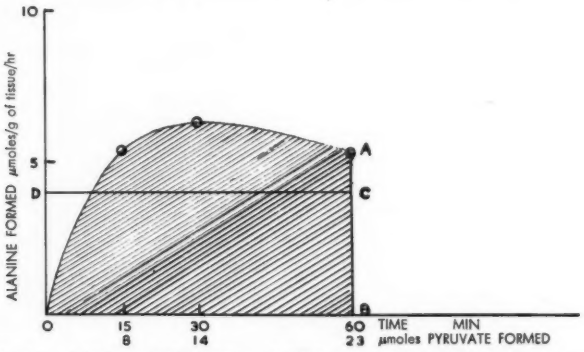

FIG. 3. Alanine yield from ABA–OA and ABA–pyruvic transaminase systems.

mation is plotted against the pyruvate concentration, the latter being superimposed on the time axis in such a way that each level of pyruvate is projected over the time required for its attainment in the course of the incubation of homogenate with OA. The rate of alanine formation at a pyruvate level of 23 μmoles (attained in 1 hour) is represented by AB, and that corresponding to 35 μmoles pyruvate by BC (on the same scale). The amount of alanine formed in 1 hour by the incubation of homogenate with ABA and pyruvate (35 μmoles) would be given by the area BCDO. On the other hand, when ABA and OA are incubated for 1 hour with the homogenate increasing amounts of pyruvate are formed which would transaminate with ABA for decreasing intervals of time and the amount of alanine formed would be represented by the area AOB (shaded). A comparison of the areas AOB and BCDO explains how ABA–OA system yields more alanine than the ABA–pyruvate system when the concentration of OA and pyruvate at the beginning of the incubation is 35 μmoles each.

Glutamic–Oxaloacetic Transaminase System

When liver homogenate was incubated with glutamate and OA (35 μmoles) on the one hand and glutamate and pyruvate (35 μmoles) on the other, it was found that the former system yielded much less alanine than the latter (Table I). This is again in conformity with our original observation (1). As in the case of ABA–OA system an attempt has been made to simulate the

glutamic–OA system by the addition of pyruvate at the same rate as it was presumably formed by the decomposition of OA on incubation with homogenate. However, the two systems yielded widely divergent amounts of alanine which was much lower in the glutamic–OA system.

In seeking an explanation for this discrepancy two possibilities have to be considered: (i) that glutamate in some way inhibits the decomposition of OA to pyruvate and (ii) that glutamate might sidetrack a good proportion of OA, thus making it unavailable for pyruvate formation. It has been possible to gain an insight into the effect of glutamate by studying the decomposition of OA and the consequent formation of pyruvate with and without added glutamate. The results are shown in Table II and Fig. 2.

It could be seen from the graph that in presence of glutamate there is a rapid disappearance of OA; but the formation of pyruvate is markedly depressed. The pronounced divergence between the plots of OA disappearance and pyruvate formation in presence of glutamate suggests that OA is in some way sidetracked, thereby diminishing its availability for pyruvate formation. With this diminution in the availability of pyruvate it is only to be expected that the alanine yield would be proportionately affected (Fig. 1). Similar studies with ABA in place of glutamate showed that ABA did not affect the conversion of OA to pyruvate.

Presumably OA is consumed in some rapid reaction in presence of glutamate and the most obvious possibility is the transamination reaction:

$$\text{glutamate} + \text{OA} \rightleftharpoons \text{aspartate} + \alpha\text{-ketoglutarate.}$$

If this assumption were correct, it should be possible to account for all the OA that disappeared during the incubation as the sum of pyruvate, alanine, and aspartate formed. It is found from Table III that the entire OA disappearance

TABLE III

Pathways of oxaloacetate disappearance in presence of glutamate

Expt. No.	OA taken, μmoles (1)	OA left unused, μmoles (2)	OA used up, μmoles (3)	Pyruvate formed, μmoles (4)	Alanine formed, μmoles (5)	Aspartate formed, μmoles (6)	Sum of columns (4), (5), and (6), μmoles (7)
1	35	3.5	31.5	8.5	7.7	14.7	30.9
2	35	2.5	32.5	10.5	8.4	15.0	33.9
3	35	2.7	32.3	11.3	6.5	15.5	33.3

could thus be accounted for. The transamination of OA with glutamate seems to be quantitatively so important that almost half of the added OA is thus used up, resulting in a marked reduction in the quantity of pyruvate made available for transamination to alanine.

OA thus seems to be disposed of by two pathways: (i) transamination to aspartate and (ii) decarboxylation to pyruvate. When ABA is used as the amino donor transamination with OA does not take place to any appreciable extent (1) and the OA is almost entirely channelled into the second pathway.

On the other hand, in the presence of glutamate OA seems to be preferentially transaminated to aspartate to such an extent that the relative magnitude of the second pathway is considerably lessened. The apparent inhibition of OA decarboxylation by glutamate referred to above is only the result of the rapid removal of a good proportion of OA through transamination.

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NOTES

THE INFLUENCE OF ETHYL ALCOHOL ON THE OXYGEN CONSUMPTION OF CEREBRAL SLICES

D. W. CLARKE AND R. L. EVANS

It has been well established that ethyl alcohol in vitro inhibits the oxygen uptake of cerebral slices incubated in a medium which contains a high concentration of potassium ions (1, 2, 3). On the other hand, if such slices are incubated in a medium which contains a physiological concentration of potassium, the addition of small amounts of ethyl alcohol stimulates, while higher concentrations inhibit the oxygen uptake (1, 2). It is the "potassium-stimulated" respiration which is apparently most sensitive to the inhibitory effects of alcohol. Most of the work in this field has been done using procedures in which alcohol is added to the slices in vitro. Few investigators have studied the influence of ethyl alcohol on the respiration of cerebral slices from animals pretreated with alcohol.

Robertson and Stewart (4) have shown that cerebral slices taken from a rabbit which had been given alcohol per rectum a short while before sacrifice had an increased oxygen uptake, if alcohol was present in the medium in which the slices were incubated. They used a Krebs–Ringer phosphate medium so the slices were not stimulated by a high concentration of potassium ions. The effect of the alcohol in the medium was variable, depending on the time which elapsed between the administration of alcohol to the animal and the preparation of the brain slice. Sometimes a stimulation and sometimes an inhibition of oxygen uptake was noted. No statistical data were given.

We have re-examined the effect of feeding of alcohol on the oxygen consumption of brain slices when they were incubated in a medium which contained a high concentration of potassium. We have also examined the effect of alcohol in the medium.

Procedure

Wistar rats of between 200 and 250 g were fed by stomach tube. either 25% w/v alcohol or water, in amounts of 3 cc/100 g body weight, 1 hour before sacrifice. Two cortical slices were obtained from each rat. One slice was incubated in a Krebs–Ringer phosphate medium to which potassium (as KCl) was added to give a final concentration of 0.1 *M*. The other slice was incubated in a similar medium but with the further addition of alcohol in the concentrations indicated. In some cases the slices were incubated in the original Krebs–Ringer medium for 1 hour, before the extra potassium was added. In other cases, the high potassium concentration was present from the start of the incubation. There was no apparent difference between the results obtained by the two methods. All incubations were carried out in a Warburg apparatus, with 2 cc of medium.

Results

The average values for the oxygen uptake of the slices, under different conditions, are shown in Table I. It is seen that alcohol in the medium depresses

TABLE I

Oxygen uptake of cerebral cortex slices in Krebs–Ringer phosphate medium (0.1 *M* KCl)

	Treated				Control			
Alcohol concn., mg/100 ml	0	125	0	500	0	125	0	500
Oxygen uptake, μl/mg/hr	0.245	0.251	0.241	0.238	0.236	0.229	0.237	0.221
Alcohol effect, μl/mg/hr	.006		−.003		−.007		−.016	
Mean value of alcohol effect ±std. error of mean	+.001 ± .0039				−.011 ± .0045			
Significance of effect	Not significant				$.025 > p > .01$			
Number of observations	14		20		17		19	

the oxygen uptake of cerebral slices taken from control animals. This agrees with the results of other investigators. On the other hand, if the rats have been pretreated with alcohol the further addition of alcohol to the medium does not inhibit the oxygen uptake, and with low concentrations of alcohol there may be some indication of a stimulating effect. There is no significant difference between the slices from treated and the slices from control rats, incubated in an alcohol-free medium, insofar as oxygen uptake is concerned.

It is difficult to compare these results with those obtained by Robertson and Stewart (4) since no statistical data were given on their charts, and their results were obtained with unstimulated brain slices, whereas the results reported here have been obtained with stimulated slices. However, the suggestion of a stimulation of oxygen uptake under the conditions mentioned above agrees with their results. Neither of the sets of data gives experimental evidence to indicate the reason for the lack of inhibitory effect of alcohol on slices taken from pretreated animals. Under the circumstances of our test the blood levels of alcohol in such animals were between 300 and 500 mg per 100 ml of blood. (Similar alcohol concentrations in vitro caused an inhibition of oxygen uptake with media which contained 0.1 *M* KCl.) Further work is planned to investigate these changes.

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CORRECTIONS

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Page 219. In Table I, for "Potassium, mM./kg.", the values 15 ± 2 (4) 20 ± 5 (5) should read 20 ± 5 (5) 15 ± 2. (4)

Pages 377, 378. On p. 377, line 25, and on p. 378, Table IV, "Galactose-U-C^{14}" should read "Galactose-1-C^{14}".

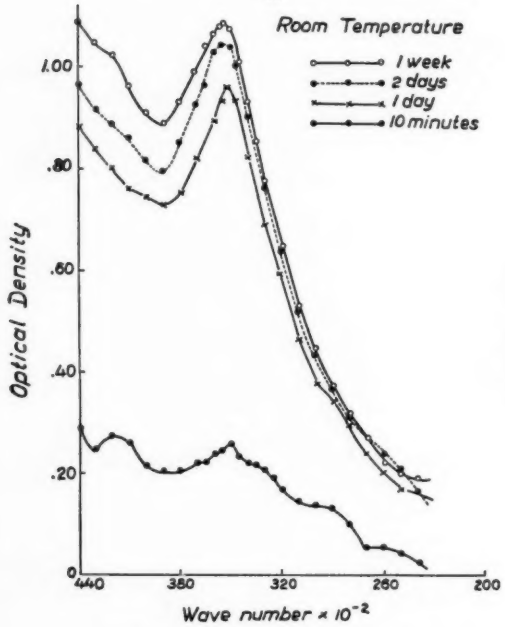
Page 959. In the Abstract, line 14, the sentence "The rate of repolarization was decreased . . ." should read "The rate of repolarization was increased . . .".

Page 1181. The first column of Table I should appear as follows:

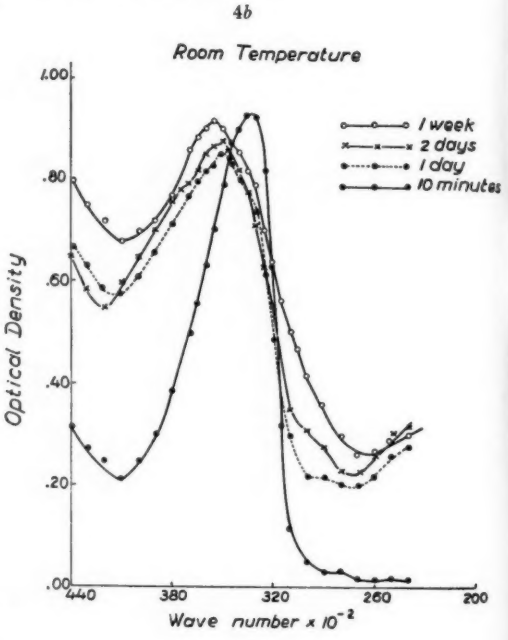
(1) Albumin* Conjugate
(2) Albumin Conjugate
(3) Albumin Conjugate
(4) Albumin Conjugate
(5) Albumin Conjugate

Pages 1227, 1228. The lower figures on these two pages should be transposed; the figure that appears on p. 1227 is 4*b* and the figure on p. 1228 is 3*b*, as indicated below.

3*b*



4*b*



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Page 28. In column 3 of Table I, opposite Experiments 1, 3, and 5, the prefix "DL-" should be omitted before the abbreviation "*p*-HPPA".

Page 289. The legend on the ordinate of Fig. 2 should read

Percent C^{14} infused per min
found per mg carbon $\times 10^{-2}$.

Page 540. In the last line, "Figure 4" should read "Figure 3."

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